

Treatment of Cancer Using Cytokine-Expressing Polynucleotides and Compositions Therefor

5 This application claims the benefit of the filing date of the following provisional applications: 60/067,087 filed November 20, 1997; 60/079,914, filed March 30, 1998; and 60/100,820, filed September 15, 1998, all of which are incorporated herein by reference. This application is a continuation-in-part of U.S. Patent Application No. 09/196,313, filed November 20, 1998, ^{now abandoned} which is incorporated herein by reference. m
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Background of the Invention

Field of the Invention

15 The present invention relates to treatment of cancer in mammals. Generally, the present invention provides methods of treating cancer in a mammal by administering a polynucleotide construct comprising a polynucleotide encoding a cytokine. In addition, the present invention relates to the methodology for selective transfection of malignant cells with polynucleotides expressing therapeutic or prophylactic molecules in intra-cavity tumor bearing mammals. More specifically, the present invention provides a methodology for the suppression of an intra-cavity dissemination of malignant cells, such as intraperitoneal dissemination.

25 The present invention further relates generally to compositions and methods useful for *in vivo* polynucleotide-based polypeptide delivery into cells of vertebrates. More particularly, the present invention provides the use of sodium phosphate solutions in compositions and methods useful for direct polynucleotide-based polypeptide delivery into the cells of vertebrates.

Related Art

Cytokines have been demonstrated both in pre-clinical animal models as well as in humans to have potent anti-tumor effects. In particular IFN's have been tried for the treatment of a number of human concerns.

The interferons (IFNs) are a family of cytokines with potent anti-viral, antiproliferative, and immunomodulatory activities and play important roles in the body's defensive response to viruses, bacteria, and tumors (Baron, S. *et al.*, *JAMA* 266:1375 (1991)). On the basis of antigenicity, biochemical properties, and producer cell, the interferon's have been divided into two classes, type I interferon and type II interferon. IFN α , IFN β , IFN ω , and IFN τ are type I interferons, and bind to the same α/β receptor. IFN γ is a type II interferon, and binds to the γ receptor (Pestka, S., *Ann. Rev. Biochem.* 56:727 (1987)). IFN α and IFN β are naturally expressed in many cells upon viral infection. IFN γ is produced by activated T lymphocytes and natural killer (NK) cells. IFN τ is believed to possess hormone activity, and plays an important role in pregnancy in cattle, sheep, and related ruminants (Imakawa, K. *et al.*, *Nature* 330:377 (1987); Stewart, H.J. *et al.*, *J. Endocrinology* 115:R13 (1987)). Due to the pleiotropic activities of IFNs, these cytokines have been studied for their therapeutic efficacy in a number of diseases, particularly cancers and viral infectious diseases.

IFN ω was discovered independently by three different groups in 1985 (Capon, D.J., *et al.*, *Molec. Cell. Biol.* 5: 768-779 (1985), Feinstein, S. *et al.*, *Molec. Cell. Biol.* 5:510 (1985); and Hauptmann and Swetly, *Nucl. Acids Res.* 13: 4739-4749 (1985)). Unlike IFN α , for which at least 14 different functional nonallelic genes have been identified in man, IFN ω is encoded by a single functional gene. IFN ω genes are believed to be present in most mammals, but have not been found in dogs, rats or mice. The mature IFN ω polypeptide is 172 amino acids and shares 60% sequence homology with the human IFN α 's. Due to the sequence similarity with IFN α , IFN ω was originally considered to be a member or a subfamily of IFN α , and was

originally termed IFN α -II. IFN ω is a significant component ($\approx 10\%$) of human leukocyte-derived interferon, the natural mixture of interferon produced after viral infection (Adolf, G. *et al.*, *Virology* 175:410 (1990)). IFN ω has been demonstrated to bind to the same α/β receptor as IFN α (Flores, I. *et al.*, *J. Biol. Chem.* 266: 19875-19877 (1991)), and to share similar biological activities with IFN α , including anti-proliferative activity against tumor cells *in vitro* (Kubes, M. *et al.*, *J. Interferon Research* 14:57 (1994) and immunomodulatory activity (Nieroda *et al.*, *Molec. Cell. Differentiation* 4: 335-351 (1996)).

Recombinant IFN α polypeptide has been approved for use in humans for hairy cell leukemia, AIDS-related Kaposi's sarcoma, malignant melanoma, chronic hepatitis B and C, chronic myelogenous leukemia, and condylomata acuminata (Baron, S. *et al.*, *JAMA* 266:1375 (1991)). However, for each of these indications, IFN α polypeptide must be administered repeatedly, often on a daily basis, for extended periods of time to maintain effective serum levels due to the short half-life (hours) of the polypeptide in the serum (Friedman, *Interferons: A Primer*, Academic Press, New York, pp. 104-107 (1981); Galvani and Cawley, *Cytokine Therapy*, Cambridge University Press, Cambridge, pp. 114-115 (1992)). Thus, in spite of producing clinical benefit for many disease conditions, the use of IFN α polypeptide is associated with acute and chronic side effects in most patients (Jones, *Cancer* 57: 1709-1715 (1986); and Quesda *et al.*, *Blood* 68: 493-497 (1986)). The severity of the adverse reaction correlates with peak serum interferon levels.

Viral or plasmid vectors containing IFN α genes have been used *ex vivo* therapy to treat mouse tumors. For example, tumor cells were transfected *in vitro* with viral or plasmid vectors containing an IFN α gene, and the transfected tumor cells were injected into mice (Belldegrun, A., *et al.*, *J. Natl. Cancer Inst.* 85: 207-216 (1993); Ferrantini, M. *et al.*, *Cancer Research* 53: 1107-1112 (1993); Ferrantini, M. *et al.*, *J. Immunology* 153: 4604-4615 (1994); Kaido, T. *et al.*, *Int. J. Cancer* 60: 221-229 (1995); Ogura, H. *et al.*, *Cancer Research* 50: 5102-5106 (1990); Santodonato, L., *et al.*, *Human Gene*

Therapy 7:1-10 (1996); Santodonato, L., *et al.*, *Gene Therapy* 4:1246-1255 (1997)). In another *ex vivo* study, cervical carcinoma and leukemia cells were transfected with a viral vector containing the interferon-consensus gene, and the transfected cells were injected into mice (Zhang, J.-F. *et al.*, *Cancer Gene Therapy* 3: 31-38 (1996)). In all of these *ex vivo* studies, varying levels of anti-tumor efficacy, such as tumor regression and/or prolonged survival, have been observed.

Viral or plasmid vectors containing interferon genes have also been used in *in vivo* therapy for tumor-bearing mice. For example, a viral vector containing the interferon-consensus gene was injected into mice bearing transplanted MDA-MB-435 breast cancer, hamster melanoma, or K562 leukemia, and tumor regression was reported (Zhang, J.-F. *et al.*, *Proc. Natl. Acad. Sci. USA* 93: 4513-4518 (1996)). In a similar study, a plasmid vector containing human IFN β gene complexed with cationic lipid was injected intracranially into mice bearing a human glioma, and tumor regression was reported (Yagi, K. *et al.*, *Biochemistry and Molecular Biology International* 32: 167-171 (1994)). In a murine model of renal cell carcinoma the direct intratumoral injection of an IL-2 plasmid DNA : lipid complex has been shown to result in complete tumor regression and a significant induction of a tumor specific CTL response increase in survival (Saffran *et al.*, *Cancer Gene Therapy* 5: 321-330 (1998)).

Plasmid vectors containing cytokine genes have also been reported to result in systemic levels of the encoded cytokine and in some cases, biological effects characteristic of each cytokine in mice. For example, the intramuscular injection of plasmid DNA encoding either TGF β , IL-2, IL-4, IL-5, or IFN α resulted in physiologically significant amounts in the systemic circulation of the corresponding cytokine polypeptide (Raz, E. *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 4523-4527 (1993); Raz, E. *et al.*, *Lupus* 4: 266-292 (1995); Tokui, M. *et al.*, *Biochem. Biophys. Res. Comm.* 233: 527-531 (1997); Lawson, C. *et al.*, *J. Interferon Cytokine Res.* 17: 255-261 (1997); Yeow, W.-S. *et al.*, *J. Immunol.* 160: 2932-2939 (1998)).

U.S. Patent No. 5,676,954 reports on the injection of genetic material, complexed with cationic liposomes carriers, into mice. U.S. Patent Nos. 4,897,355; 4,946,787; 5,049,386; 5,459,127; 5,589,466; 5,693,622; 5,580,859; 5,703,055; and International Patent Application No. PCT/US94/06069 (publication no. WO 94/29469) provide cationic lipids for use in transfecting DNA into cells and mammals. U.S. Patent Nos. 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international patent application no. PCT/US94/06069 (publication no. WO 04/9469) provide methods for delivering DNA-cationic lipid complexes to mammals.

Even though some viral vectors used in *ex vivo* and *in vivo* cancer therapy in murine models showed anti-tumor efficacy, the use of viral vectors to deliver interferon-expressing genes in vivo could induce anti-viral immune responses or result in viral integration into host chromosomes, causing disruption of essential host genes or activation of oncogenes (Ross *et al.*, *Human Gene Therapy* 7: 1781-1790 (1996)).

For treatment of multiple metastatic carcinomas of a body cavity are treated using laparoscopy (Childers *et al*, *Gynecol. Oncol.* 59: 25-33. (1995)), catheterization (Naumann *et al*, *Gynecol. Oncol.* 50: 291-3, (1993)) or other access devices (Almadrones *et al*, *Semin. Oncol. Nurs.* 11: 194-202, (1995)). Treatment is usually by surgical removal of primary and large metastatic tumors and postoperative chemotherapy (Kigwawa *et al*, *Am. J. Clin. Oncol.* 17: 230-3, (1994); Markman *et al*, *J. Clin. Oncol.* 10: 1485-91, (1992)) or radiotherapy (Fjeld *et al*, *Acta. Obstet. Gynecol. Scand. Suppl.* 155: 105-11, (1992)). Tumor recurrence is monitored by magnetic resonance imaging (Forstner *et al*, *Radiology* 196: 715-20, (1995)), ascites cytology (Clement, *Am. J. Clin. Pathol.* 103: 673-6, (1995); Forstner *et al*, *Radiology* 196: 715-20, 1995) and blood analyses (Forstner *et al*, *Radiology* 196: 715-20, (1995)). Many intraperitoneal (i.p.) cancers, such as ovarian cancer, eventually metastasize via the lymphatic system to the lungs or other vital organs, and the prognosis for the patient is very poor (Kataoka *et al*, *Nippon Sanka Fujinka Gakkai Zasshi* 46: 337-44, 1994; Hamilton, *Curr. Probl. Cancer* 16: 1-57, (1992)).

Human ovarian cancer is often diagnosed at an advanced stage when the effectiveness of surgery and chemotherapy are limited. The lack of effective treatment options for late-stage patients warrants the development of new treatment modalities for this disease. There have been several attempts to develop an effective immunotherapy for the treatment of ovarian cancer.

The early work in this area involved mouse studies in which bacteria-derived immunostimulants, such as *Bacillus Calmette-Guerin* (BCG) and *Corynebacterium parvum*, were injected i.p. as non-specific activators of the immune system. (Knapp and Berkowitz, *Am. J. Obstet. Gynecol.*, 128: 782-786, (1977); Bast *et al.*, *J. Immunol.*, 123: 1945-1951, (1979); Vanhaelen, *et al.*, *Cancer Research*, 41: 980-983, (1981); and Berek, *et al.*, *Cancer Research*, 44, 1871-1875, (1984)). These studies generally resulted in a non-specific immune response that often did not prevent the growth of later tumors. In addition, if the bacterial antigens were injected more than 24 hours after tumor cell inoculation, there was minimal antitumor response, suggesting that treatment of late-stage ovarian cancer patients with this type of therapy would not be effective.

More recent studies in both mice and humans have involved the i.p. or intravenous (i.v.) administration of cytokine proteins as more specific activators of the immune response (Adachi, *et al.*, *Cancer Immunol. Immunother.* 37: 1-6, (1993); Lissoni, *et al.*, *Tumori*. 78: 118-20, (1992)). Treating murine ovarian tumors with a combination of recombinant IL-2 and GM-CSF proteins had some beneficial effect in inhibiting ascites production; however, IL-2 was only effective if it was combined with GM-CSF (Kikuchi, *et al.*, *Cancer Immunol. Immunother.*, 43: 257-261, (1996)). Similarly, a combination of IL-2 and lymphokine-activated killer (LAK) cells was able to reduce i.p. sarcomas in mice, while IL-2 protein alone was not as effective (Ottow, *et al.*, *Cellular Immunology*, 104: 366-376, (1987)). Human clinical trials evaluating IL-2 protein therapy of ovarian cancer patients indicated some antitumor effects (Chapman *et al.*, *Investigational New Drugs*, 6:179-188, (1988); West *et al.*, *N. Engl. J. Med.* 316:898-905, 1987; Lotze *et al.*, *Arch. Surg.* 121:1373-1379, 1986; Benedetti Panici *et al.*, *Cancer Treatment Review*,

16A:123-127, 1989; Beller *et al.*, *Gynecol. Oncol.*, 34:407-412, 1989; Urba *et al.*, *J. Natl. Cancer Inst.*, 81:602-611, 1989; Stewart *et al.*, *Cancer Res.*, 50:6302-6310, 1990; Steis *et al.*, *J. Clin. Oncol.*, 8:1618-1629, 1990; Lissoni *et al.*, *Tumori*, 78:118-120, 1992; Sparano *et al.*, *J. of Immunotherapy*, 16:216-223, 1994; Freedman *et al.*, *J. of Immunotherapy*, 16:198-210, 1994; Edwards *et al.*, *J. Clin. Oncol.*, 15:3399-3407, 1997).

Recent studies in mice have involved the injection of DNA constructs encoding "suicide" genes followed by treatment with prodrugs. This approach has successfully caused regression of some small tumors but has been less successful on larger tumor masses. (Szala, *et al. Gene Therapy* 3: 1025-1031, 1996; Sugaya, *et al. Hum Gene Ther* 7: 223-230 (1996)). In another study, liposome-mediated *E1A* gene therapy for mice bearing ovarian cancers that overexpress HER-2/*neu* resulted in reduced mortality among these tumor-bearing mice. (Yu, *et al. Oncogene*, 11: 1383-1388 (1995)). Similarly, the successful treatment of murine ovarian carcinoma (MOT) has been demonstrated using cisplatin-induced gene transfer of DNA constructs encoding IFN γ via i.p. injection. (Son, *Cancer Gene Therapy* 4: 391-396 (1997)). However, this study demonstrated that tumors were poorly responsive to either the IFN γ gene or cisplatin alone, suggesting that the effectiveness of the cisplatin-based gene therapy protocol was mainly due to enhanced sensitization of cisplatin-exposed tumor cells to transfection by the IFN γ gene. (Son, *Cancer Gene Therapy* 4: 391-396, 1997).

Clearly, there is a need for superior therapeutic compositions and methods for treating mammalian cancer. Further, there is a need for an *in vivo* delivery system for IFN ω . The present invention provides a simple and safe yet effective compositions and methods for treatment of mammalian cancer.

The present invention also solves the problems inherent in prior attempts to treat body cavity malignancies. The inventors show herein that the malignant cell dissemination into body cavities, such as into the peritoneal cavity during late stage ovarian cancer, can be suppressed simply by administering as few as two to six doses of a polynucleotide formulation directly into the body cavity. This treatment results in selective transfection of

malignant cells, and subsequent long-term local production of an effective amount of therapeutic molecules.

The *in vivo* delivery of a polynucleotide (e.g., plasmid DNA) into vertebrate tissues has been shown to result in the cellular uptake and expression of the polynucleotide into a desired polypeptide (Wolff, J.A. *et al.*, *Science* 247:1465-1468 (1990); Wheeler, C.J. *et al.*, *Proc. Natl. Acad. Sci. USA* 93:11454-11459 (1996)). Potential human therapeutic uses of such polynucleotide-based polypeptide delivery include immune response induction and modulation, therapeutic polypeptide delivery, and amelioration of genetic defects. For example, a polynucleotide may encode an antigen that induces an immune response against an infectious pathogen or against tumor cells (Restifo, N.P. *et al.*, *Folia Biol.* 40:74-88 (1994); Ulmer, J.B. *et al.*, *Ann. NY Acad. Sci.* 772:117-125 (1995); Horton, H.M. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:1553-1558 (1999); Yagi, K. *et al.*, *Hum. Gene Ther.* 10: 1975-1982 (1999)). The polynucleotide may encode an immunomodulatory polypeptide, e.g., a cytokine, that diminishes an immune response against self antigens or modifies the immune response to foreign antigens, allergens, or transplanted tissues (Qin, L. *et al.*, *Ann. Surg.* 220:508-518 (1994); Dalesandro, J. *et al.*, *J. Thorac. Cardiovasc. Surg.* 111: 416-421 (1996); Moffatt, M. and Cookson, W., *Nat. Med.* 2:515-516 (1996); Ragno, S. *et al.*, *Arth. and Rheum.* 40:277-283 (1997); Dow, S.W. *et al.*, *Hum. Gene Ther.* 10:1905-1914 (1999); Piccirillo, C.A. *et al.*, *J. Immunol.* 161:3950-3956 (1998); Piccirillo, C.A. and Prud'homme, G.J., *Hum. Gene Ther.* 10: 915-1922 (1999)). For therapeutic polypeptide delivery, the polynucleotide may encode, for example, an angiogenic protein, hormone, growth factor, or enzyme (Levy, M.Y. *et al.*, *Gene Ther.* 3:201-211 (1996); Tripathy, S.K. *et al.*, *Proc. Natl. Acad. Sci. USA* 93:10876-10880 (1996); Tsurumi, Y. *et al.*, *Circulation* 94:3281-3290 (1996); Novo, F.J. *et al.*, *Gene Ther.* 4:488-492 (1997); Baumgartner, I. *et al.*, *Circulation* 97:1114-1123 (1998); Mir, L.M. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:4262-4267 (1999)). For amelioration of genetic defects, the polynucleotide may encode normal copies of defective proteins such as dystrophin or cystic fibrosis transmembrane conductance regulator (Danko, I. *et al.*, *Hum. Mol.*

Genet. 2:2055-2061 (1993); Cheng, S.H. and Scheule, R.K., *Adv. Drug Deliv. Rev.* 30:173-184 (1998)).

However, the efficiency of a polynucleotide uptake and expression, especially when the polynucleotide is not associated with infectious agents, is relatively low. For example, Doh, S.G. *et al.*, *Gene Ther.* 4:648-663 (1997) reports that the administration of plasmid DNA into mouse muscle results in the detectable transduction of an average of only 6%, *e.g.*, about 234 out of approximately 4000, of the myofibers in the injected muscle. Wheeler, C.G. *et al.*, *ibid.*, showed that administration of plasmid DNA complexed with cationic lipid into a mouse lung results in the transduction of less than 1% of the lung cells.

Attempts have been made to increase the efficiency of *in vivo* polynucleotide administration into vertebrates using chemical agents or physical manipulations. Such chemical agents include cellular toxins such as bupivacaine or barium chloride (Wells, D.J., *FEBS Letters* 332:179-182 (1993); Vitadello, M. *et al.*, *Hum. Gene Ther.* 5:11-18 (1994); Danko, I. *et al.*, *Hum. Mol. Genet.* 2:2055-2061 (1993)) which act to cause muscle damage followed by muscle regeneration by cell division which makes the cells more receptive to DNA entry (Thomason, D.B. and Booth, F.W., *Am. J. Physiol.* 258:C578-81 (1990)); polymers such as polyvinyl pyrrolidone that coat the DNA and protect it from DNases (Mumper, R.J., *et al.*, *Pharm. Res.* 13:701-709 (1996); Mumper R. J. *et al.*, *J. Cont. Rel.* 52:191-203 (1998); Anwer, K. *et al.*, *Pharm. Res.* 16:889-95 (1999)); bulking agents such as sucrose that are injected before DNA injection to help expand the spaces between muscle cells and therefore allow better distribution of the subsequently injected DNA (Davis, H.L. *et al.*, *Hum. Gene Ther.* 4:151-159 (1993)); DNA binding agents such as histones or intercalators that protect the DNA from DNases (Manthorpe, M. *et al.*, *Hum. Gene Ther.* 4:419-431 (1993); Wolff, J.A., *Neuromuscul. Disord.* 7:314-318 (1997); WO 99/31262). Physical manipulations include removal of nerves that control muscle contraction (Wolff, J.A. *et al.*, *BioTechniques* 11:575-485 (1991)); electroporation that electrically opens muscle cell pores allowing more DNA entry (Aihara, H. and

Miyazaki, J., *Nature Biotechnol.* 16:867-870 (1998); Mir, L.M. *et al.* *CR Acad. Sci. III* 321:893-899 (1998), Mir, L.M. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:4262-4267 (1999); Mathiesen, I., *Gene Ther.* 6:508-514 (1999); Rizzuto, G. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:6417-6422 (1999)); use of
5 intravascular pressure (Budker, V. *et al.*, *Gene Ther.* 5:272-276 (1998)); use of
sutures coated with plasmid DNA (Labhasetwar, V. *et al.*, *J. Pharm. Sci.*
87:1347-1350 (1998); Qin, Y. *et al.*, *Life Sci.* 65:2193-2203 (1999)); use of
sponges soaked with DNA as intramuscular depots to prolong DNA delivery
(Wolff, J.A. *et al.* (1991), *ibid.*); use of special needle-based injection methods
10 (Levy, M.Y. *et al.*, *Gene Ther.* 3:201-211 (1996); Doh, S.G. *et al.* (1997),
ibid.); and of needleless-injectors that propel the DNA into cells (Gramzinski,
R.A. *et al.*, *Molec. Med.* 4:109-118 (1998); Smith, B.F. *et al.*, *Gene Ther.*
5:865-868 (1998); Anwer, K. *et al.* (1999) *ibid.*). In addition, Wolff, J.A. *et al.*
(1991) *ibid.* and Manthorpe, M. *et al.* (1993) *ibid.* refers to conditions
15 affecting direct gene transfer into rodent muscle *in vivo*.

WO99/64615 identifies the use of products and methods useful for
delivering formulated nucleic acid molecules using electrical pulse voltage
delivery. Examples include the formulation of plasmid DNA in a saline
solution containing agents that promote better delivery of the plasmid DNA
20 into cells *in vivo* when the formulation is delivered with an electrical pulse.
Electrical pulse delivery often comprises electroporation where an electrical
pulse is delivered to a tissue that is previously injected with a drug.
Electroporation of a tissue causes transient interruption of cell membranes
allowing more drug to enter the cell through the interruptions or "pores". The
25 agents in the saline DNA solution that promote delivery of the DNA into
electroporated tissues include propylene glycols, polyethylene glycols,
poloxamers (block copolymers of propylene oxide and ethylene oxide), or
cationic lipids. They claim that the way that these agents enhance delivery of
the DNA into cells is by either protecting the DNA from degradation by
30 DNases or by condensing the DNA into a smaller form, or both.

Many of these attempts to enhance tissue transduction have used agents that destroy muscle (bupivacaine, barium chloride) and actually lower expression (Norman, J. *et al.*, *Methods in Molec. Med.* 29:185-196 (1999)); have to be pre-injected before the DNA (sucrose); are expensive organic polymers (polyvinyl pyrrolidone), mutagens (intercalators), antigenic proteins (histones) or devices that destroy muscle tissue (needleless or needle-free injectors); or need to be inserted surgically (sutures, sponges, intravascular pressure). Furthermore, most of these methods may be expensive and not suitable or practical for human use.

On the other hand, little attention has been given to the use of alternative salt solutions and/or auxiliary agents in the pharmaceutical formulation as a way of enhancing the efficiency of a polynucleotide-based polypeptide delivery. Investigators in this field routinely use normal saline or phosphate buffered saline (PBS 0.9% (*i.e.*, about 154 mM) NaCl and 10 mM Na-phosphate) solutions for polynucleotide delivery, *e.g.*, by intramuscular injection, because they are physiologically isotonic, isoosmotic, stable, non-toxic, and also because they have been traditionally used for human intramuscular injections of other drugs. However, sodium phosphate, in the absence of saline, has been used in humans for delivery of non-polynucleotide-based drugs (*e.g.*, small molecules) administered via the intramuscular or intravenous routes (*See generally, Physician's Desk Reference.* Medical Economics Co, Monynvale, New Jersey (1998)).

Sodium or potassium phosphate have been reported to enhance LipofectinTM-mediated transfection of human osteosarcoma cells *in vitro* (Kariko, K., *et al.*, *Biochim Biophys Acta* 1369:320-334 (1998)), and the use of RPMI cell culture medium buffered with NaHCO₃/Na₂HPO₄ were reported to be the best medium for forming DNA/cationic lipid complexes *in vitro*. (Kichler, A., *et al.*, *Gene Ther.* 5:855-860 (1998)).

There remains a need in the art for a convenient and safe way of improving the effectiveness of *in vivo* polypeptide delivery via direct administration of a polynucleotide. Aqueous solutions of certain salts including sodium phosphate have been used in humans (*i.e.*, intramuscular

injection of various small molecule drugs), and detergents or surfactants as auxiliary agents are common additives in drugs administered into human tissues. However, the use of certain salts or auxiliary agents, or a combination thereof to improve the transduction, *i.e.*, the entry into cells, and/or expression-enhancing efficiency of polynucleotides delivered *in vivo* is new.

Summary of the Invention

The present invention is broadly directed to treatment of cancer by administering *in vivo*, into a tissue of a mammal suffering from cancer, a polynucleotide construct comprising a polynucleotide encoding a cytokine. The polynucleotide construct is incorporated into the cells of the mammal *in vivo*, and a therapeutically effective amount of a cytokine is produced *in vivo*, and delivered to tumor cells. Combinations of cytokine-encoding polynucleotides can be administered.

The present invention provides a pharmaceutical composition comprising about 1 ng to 20 mg of a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide selected from the group consisting of (a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; (b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and (c) a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and any of the above group complexed with one or more cationic compounds selected

from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

The present invention also provides a pharmaceutical composition obtained by complexing a polynucleotide selected from the group consisting of (a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; (b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; (c) a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, with one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

The present invention also provides a method of treating cancer in a mammal, comprising administering into a tissue of the mammal a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide encoding a cytokine, or an active fragment thereof, such that the polynucleotide is expressed *in vivo*, and such that the cytokine, or active fragment thereof, is delivered systemically to a tumor tissue in an amount effective to treat the cancer.

The present invention also provides a method of treating cancer in a mammal, comprising administering into a tissue of the mammal a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide encoding a cytokine selected from the group consisting of interferon- ω , interferon- α , and a combination thereof, such that the polynucleotide or an active fragment thereof is expressed, and such that the cytokine is delivered locally to a tumor tissue in an amount effective to treat

the cancer. Preferably, the polynucleotide construct is complexed with a cationic vehicle, more preferably, the cationic vehicle may be a cationic lipid, and most preferably, the cationic lipid may be mixed with a neutral lipid.

Another object of the invention is to provide a method of selectively
5 transfecting malignant cells in a body cavity of a tumor-bearing mammal, comprising administering into the body cavity at least one non-infectious, non-integrating polynucleotide complexed with a cationic vehicle, such that the polynucleotide is expressed substantially in the malignant cells of the body cavity. Preferably, the cationic vehicle comprises one or more cationic lipids,
10 and more preferably, the cationic vehicle comprises a cationic and neutral lipid mixture. In a preferred embodiment, the present invention is used to suppress peritoneal dissemination of malignant cells in a tumor-bearing mammal. In particular, the mammal may have ovarian cancer, or metastasis of ovarian cancer. Preferred polynucleotides may encode cytokines, or active fragments thereof. Most preferably, the polynucleotide may encode IL-2, or an active
15 fragment thereof.

Compared to injection of recombinant cytokine polypeptides, the methods described herein have several important advantages. The present invention shows that *in vivo* transfection of cells with encoding
20 polynucleotide, such as an IL-2 or IFN γ , results in serum levels of the corresponding cytokine that have therapeutic effects, and yet are lower than the maximal serum levels typically required when cytokine polypeptides are injected. Further, injecting frequent high doses of cytokine polypeptides can produce debilitating side effects. The methods of the present invention
25 provide cytokine therapy requiring less frequent injections of cytokine-encoding nucleic acids. The injection of polynucleotide constructs encoding cytokines produces sustained, low levels of biologically active cytokines that have beneficial effects, while minimizing adverse side effects.

Compared to the delivery of cytokine genes via a viral gene delivery
30 vectors, the present method also has important advantages. Injection of non-viral vectors of the present method does not induce significant toxicity or pathological immune responses, as described, for example, in mice, pigs or

monkeys (Parker, *et al.*, *Human Gene Therapy* 6: 575-590 (1995); and San, *et al.*, *Human Gene Therapy* 4: 781-788 (1993)). Thus, a non-viral vector is safer and can be repeatedly injected.

5 The present invention is further directed to compositions, and methods for using such compositions, for improving the effectiveness of polypeptide delivery into a vertebrate by administering *in vivo*, a polypeptide-encoding polynucleotide in an aqueous solution sodium phosphate. The polynucleotide is incorporated into the cells of the vertebrate *in vivo*, and encodes a detectable amount or a prophylactically or therapeutically effective amount, of a desired
10 polypeptide.

The present invention further provides a composition selected from the group consisting of (a) a composition comprising about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; and sodium phosphate dissolved in the aqueous solution at a molar concentration from about 20 mM to about 300 mM, and reaction, association, or dissociation products thereof;
15 and (b) a composition comprising: about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; sodium phosphate dissolved in the aqueous solution at a molar concentration from about 0.1 mM to about 150 mM, and reaction, association, or dissociation products thereof;
20 and a cationic lipid suspended in said aqueous solution; where the aqueous solution is substantially free of chloride anion.

Another aspect of the present invention is a method for delivering a
25 polypeptide into a vertebrate, comprising administering to the vertebrate a composition selected from the group consisting of (a) a composition comprising about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; and sodium phosphate dissolved in the aqueous solution at a
30 molar concentration from about 20 mM to about 300 mM, and reaction, association, or dissociation products thereof; and (b) a composition comprising: about 1 ng to about 30 mg of a polynucleotide in aqueous

solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; sodium phosphate dissolved in the aqueous solution at a molar concentration from about 0.1 mM to about 150 mM, and reaction, association, or dissociation products thereof; and a cationic lipid suspended in said aqueous solution; where the aqueous solution is substantially free of chloride anion; such that the polypeptide encoded by the delivered polynucleotide is expressed in the vertebrate, in an amount sufficient to be detectable.

Another aspect of the present invention is a method for delivering a therapeutic polypeptide into a vertebrate, comprising administering to a vertebrate in need of such a therapeutic polypeptide a composition selected from the group consisting of (a) a composition comprising about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; and sodium phosphate dissolved in the aqueous solution at a molar concentration from about 20 mM to about 300 mM, and reaction, association, or dissociation products thereof; and (b) a composition comprising: about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; sodium phosphate dissolved in the aqueous solution at a molar concentration from about 0.1 mM to about 150 mM. and reaction, association, or dissociation products thereof; and a cationic lipid suspended in said aqueous solution; where the aqueous solution is substantially free of chloride anion; such that a therapeutic polypeptide encoded by the delivered polynucleotide is expressed in the vertebrate, in a therapeutically effective amount.

The present invention also provides a method of producing antibodies to a polypeptide in a vertebrate, comprising administering to the vertebrate a composition selected from the group consisting of (a) a composition comprising about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; and sodium phosphate dissolved in the aqueous solution at a molar concentration from about 20 mM to about 300 mM, and reaction,

association, or dissociation products thereof; and (b) a composition comprising: about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; sodium phosphate dissolved in the aqueous solution at
5 a molar concentration from about 0.1 mM to about 150 mM, and reaction, association, or dissociation products thereof; and a cationic lipid suspended in said aqueous solution; where the aqueous solution is substantially free of chloride anion; such that a polypeptide encoded by the delivered polynucleotide is expressed in the vertebrate, in a sufficient amount to
10 generate antibody to the encoded polypeptide in the vertebrate.

The present invention also provides a method of enhancing or modulating an immune response in a vertebrate in need of such an enhanced or modulated immune response, comprising administering to the vertebrate a composition selected from the group consisting of (a) a composition
15 comprising about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; and sodium phosphate dissolved in the aqueous solution at a molar concentration from about 20 mM to about 300 mM, and reaction, association, or dissociation products thereof; and (b) a composition
20 comprising: about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; sodium phosphate dissolved in the aqueous solution at a molar concentration from about 0.1 mM to about 150 mM, and reaction, association, or dissociation products thereof; and a cationic lipid suspended in
25 said aqueous solution; where the aqueous solution is substantially free of chloride anion; such that an immunogenic and/or immunomodulatory polypeptide encoded by the delivered polynucleotide is expressed in the vertebrate, in a sufficient amount to induce a desired immune response in the vertebrate to prevent disease or treat disease, *i.e.*, cure disease, reduce the
30 severity of disease symptoms, or prolong the life of the vertebrate.

The invention further provides a method of delivering a physiologically or metabolically necessary polypeptide to a vertebrate

incapable of making a functional form of the polypeptide, comprising administering to the vertebrate a composition selected from the group consisting of (a) a composition comprising about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably
5 encodes a polypeptide within vertebrate cells *in vivo*; and sodium phosphate dissolved in the aqueous solution at a molar concentration from about 20 mM to about 300 mM, and reaction, association, or dissociation products thereof; and (b) a composition comprising: about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably
10 encodes a polypeptide within vertebrate cells *in vivo*; sodium phosphate dissolved in the aqueous solution at a molar concentration from about 0.1 mM to about 150 mM, and reaction, association, or dissociation products thereof; and a cationic lipid suspended in said aqueous solution; where the aqueous solution is substantially free of chloride anion; such that a functional self polypeptide, *i.e.*, a physiologically or metabolically necessary polypeptide
15 encoded by the delivered polynucleotide is expressed in the vertebrate, in a sufficient amount to supply the vertebrate's requirements for the polypeptides.

The present invention also provides a pharmaceutical kit selected from the group consisting of: (a) a pharmaceutical kit comprising: a container or
20 containers holding about 1 ng to about 30 mg of a polynucleotide which operably encodes a polypeptide within vertebrate cells *in vivo*; an amount of sodium phosphate which, when dissolved in a prescribed volume of distilled water, results in an aqueous solution with a molar concentration of sodium phosphate from about 20 mM to about 300 mM, and reaction, association, or
25 dissociation products thereof; and optionally, an administration means and/or an instruction sheet; whereby the polynucleotide is provided in a prophylactically or therapeutically effective amount to treat a vertebrate; and (b) a pharmaceutical kit comprising: a container or containers holding about 1 ng to about 30 mg of a polynucleotide which operably encodes a polypeptide
30 within vertebrate cells *in vivo*; an amount of sodium phosphate which, when dissolved in a prescribed volume of distilled water, results in an aqueous solution with a molar concentration of said salt from about 0.1 mM to about

150 mM, and reaction, association, or dissociation products thereof, and where the aqueous solution formed thereby is essentially free of chloride anion; a cationic lipid; and optionally, an administration means and/or an instruction sheet; whereby the polynucleotide is provided in a prophylactically or therapeutically effective amount. Any of components of the pharmaceutical kit can be provided in a single container, or in multiple containers packaged together.

The inventors have discovered that delivery of the compositions provided herein to a vertebrate results in much improved *in vivo* polypeptide expression over the delivery of existing nucleic acid-based compositions, *e.g.*, compositions comprising polynucleotides which encode a polypeptide and an aqueous solution consisting of sterile water, normal saline (*i.e.*, 154 mM sodium chloride), or phosphate buffered saline (*i.e.*, 154 mM sodium chloride plus 10 mM sodium phosphate).

Brief Description of the Figures

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same become better understood by reference to the following detailed description when considered in connection with the accompanying figures.

FIG. 1 shows the plasmid map of VR4151 (SEQ ID No. 4). The cytomegalovirus immediate-early gene promoter enhancer and 5' untranslated sequences (5'UTR + intron A) drive the expression of the human interferon ω coding sequence. The transcriptional terminator region includes polyadenylation and termination signals derived from the rabbit β -globin gene.

FIG. ^{2A and 2B} 2 shows the pharmacokinetics of hIFN ω in the serum of C57BL/6 mice (FIG. 2A) and nude mice (FIG. 2B) after a single intramuscular (i.m.) injection of hIFN ω plasmid DNA (VR4151). Mice were injected i.m. with 100 μ g of VR4151. Following the intramuscular injection, mice were bled daily, and serum was collected and assayed for

hIFN ω polypeptide using an ELISA. Each point represents an average of four mice. In C57BL/6 mice, the single i.m. injection resulted in peak serum levels of 254 pg/ml on day 6 after injection, and serum levels were still detectable 14 days after injection (50 pg/ml) (FIG. 2A). In nude mice, the single i.m. injection resulted in peak serum levels of 648 pg/ml on day 7, and serum levels were still detectable 14 days after injection (134 pg/ml) (FIG. 2B).

3A-3F

FIG. 3 shows that systemic mIFN α treatment reduces tumor volume (FIGs. 3A, 3C, and 3E) and increases survival (FIGs. 3B, 3D, and 3F) in three murine tumor models. C57BL/6 mice bearing subcutaneous B16F10 melanoma (FIGs. 3A and 3B), subcutaneous glioma 261 (FIGs. 3C and 3D), or DBA/2 mice bearing subcutaneous Cloudman melanoma (FIGs. 3E and 3F) were injected with 100 μ g either of VR4111 (mIFN α plasmid) or VR1055 (control plasmid), twice per week for three weeks, beginning on day 4 after tumor cell injection (n=8-10 mice per group).

4A and 4B

FIG. 4 shows that systemic mIFN α , mIL-2 or mIL-12 plasmid DNA treatment reduces tumor volume (FIG. 4A) and mIFN α or mIL-12 plasmid DNA treatment increases survival (FIG. 4B) in the subcutaneous B16F10 melanoma model. C57BL/6 mice bearing subcutaneous B16F10 melanoma were injected with 100 μ g of VR4111 (mIFN α), VR4001 (mIL-12), VR1110 (mIL-2), or VR1012 (control plasmid) (n = 15-16 mice per group) twice per week for three weeks.

5A and 5B

FIG. 5 shows that i.m. administration of hIFN ω pDNA reduces tumor volume (FIG. 5A) and increases survival (FIG. 5B) in nude mice bearing human A431 epidermoid carcinoma tumors. Mice bearing human A431 tumors between 30-80 mm³ were injected i.m. with 200 μ g of either VR4151 (hIFN ω plasmid) or VR1055 (control plasmid) twice per week for three weeks (n=15).

FIG. 6 shows that i.m. administration of mIFN α pDNA reduces B16F10 melanoma lung metastases in C57BL/6 mice. Mice bearing lung metastases of B16F10 melanoma were injected i.m. with 100 μ g of either

VR4111 or VR1055 twice per week for three weeks, beginning on day 4 after tumor cell injection (n=10 mice per group). "TNTC" means too numerous to count as seen in the control group.

FIG. ^{7A and 7B} 7 shows that i.m. administration of mIFN α pDNA reduces intradermal M5076 primary tumor growth (FIG. 7A) as well as liver metastases (FIG. 7B) in C57BL/6 mice bearing murine M5076 reticulum cell sarcoma cells. Mice bearing M5076 tumors were injected i.m. with 100 μ g of either VR4111 or VR1055 twice per week for three weeks, beginning on day 4 after tumor cell inoculation (n=10-13 mice per group).

FIG. ^{8A-8D} 8 shows a comparison of different dosages and frequencies of mIFN α pDNA administration in the subcutaneous B16F10 melanoma model. C57BL/6 mice bearing subcutaneous B16F10 melanoma were injected i.m. with 50 μ g or 100 μ g of either VR4111 or VR1055 twice a week for 3 weeks beginning 4 days after tumor cell inoculation (n=10 mice per group). All groups treated with 100 μ g of VR4111 showed significant reduction in tumor growth by day 21 (p=0.002) and significant enhancement in survival (p<0.008) with all treatments tested (FIGS. 8A and 8B). In mice treated with 50 μ g VR4111, tumor growth was significantly reduced by day 21 (p=0.005), and survival was significantly increased (p<0.003) in the groups of mice that were injected twice per week or once per week. The group injected every other week with 50 μ g VR4111 was not significantly different from the mice that received the control plasmid (FIGS. 8C and 8D).

FIG. ^{9A-9D} 9 shows the results of experiments performed to determine the role of NK and T cells in the antitumor response induced by mIFN α plasmid DNA. Nude mice (T cell deficient) (FIGS. 9A and 9B), and beige-nude mice (NK and T cell deficient) (FIGS. 9C and 9D) bearing subcutaneous B16F10 melanoma tumors were injected i.m. with 100 μ g of either VR4111 or VR1055 twice per week for three weeks, beginning on day 4 after tumor cell injection (n=15 mice per group). No significant reduction in tumor volume or increase in survival was found for nude or nude-beige mice treated with

VR4111, suggesting that T cells are involved in the mIFN α antitumor response.

10A and 10B

FIG. 10 shows the results of experiments performed to evaluate the role of CD4⁺ and CD8⁺ T cells in the mIFN α DNA antitumor response. For depletion of CD4⁺ and CD8⁺ T cells, C57BL/6 mice bearing subcutaneous B16F10 melanoma tumors were injected i.p. with 500 μ g of either the anti-CD4 mAb (clone GK1.5, rat IgG) (ATCC, Rockville, MD) or anti-CD8 mAb (clone 2.43, rat IgG) (ATCC, Rockville, MD) one day after each i.m. injection of 100 μ g of either VR4111 or VR1055 twice per week for three weeks (n = 10 mice per group). The mIFN α plasmid DNA therapy significantly reduced tumor growth ($p \leq 0.002$) and enhanced survival ($p \leq 0.008$) of both normal mice and mice depleted of CD4⁺ T cells, suggesting that CD4⁺ T cells were not required for the response. In contrast, mice depleted of CD8⁺ T cells and injected with VR4111 had tumor volumes and survival that were not significantly different from mice treated with the control plasmid DNA, indicating a requirement for CD8⁺ T cells in the antitumor response.

11A and 11B

FIG. 11 shows that intratumoral hIFN ω (VR4151) and hIFN α (VR4112) treatment reduces tumor volume in the human A375 melanoma model (FIG. 11A) and human NIH-OVCAR3 (FIG. 11B) in nude mice. Mice bearing subcutaneous tumor received direct intratumoral injections of a complex of DNA:DMRIE/DOPE (1:1 DNA:lipid mass ratio, 100 μ g of plasmid DNA) for 6 consecutive days followed by an additional 5 treatments every other day for a total of 11 injections (A375 melanoma model), or for every other day for a total of 11 injections (NIH-OVCAR3 ovarian cancer model).

12A and 12B

FIG. 12 shows that intratumoral mIFN α (VR4101) plasmid DNA treatment reduces tumor volume (FIG. 12A) and increases survival (FIG. 12B) in the subcutaneous B16F10 melanoma model in C57BL/6 mice. Mice received a subcutaneous implantation of 10⁴ B16F10 cells into the flank. Beginning at day 12 post tumor implant, mice received six consecutive

intratumoral injections of a complex of pDNA:DMRIE/DOPE (1:1 DNA:DMRIE mass ratio, 100 µg of plasmid DNA).

FIG. ^{13A and 13B} ~~13~~ shows luciferase activity in peritoneal tissues and MOT ascites in mice after i.p. injection of luciferase DNA:lipid complex. The results show high levels of reporter gene expression in ascites but low levels in peritoneal tissue. MOT tumor-bearing C3H/HeN mice received i.p. injections of a complex of pDNA:DMRIE/DOPE (1:1 DNA:DMRIE mass ratio, 100 µg of plasmid DNA) on days 5 and 6 after tumor cell implant. Tissues were collected 1 day (FIG. 13A) or 3 days (FIG. 13B) following the DNA:lipid injection.

FIG. ^{14A and 14B} ~~14~~ shows serum levels of IL-2 after i.p. injection of either IL-2 pDNA or protein in MOT tumor bearing mice. The serum levels of IL-2 were much lower than levels in ascites. Ascites and serum were collected at 4 hours and days 1, 2, 3, 6 and 10 post DNA or protein injection (5 mice for each time point), and analyzed for mIL-2 polypeptide using an ELISA.

FIG. ^{15A-15F} ~~15~~ shows a significant reduction in MOT tumor growth ($p=0.01$) (FIG. 15A) and increased survival ($p=0.04$) (FIG. 15B) of mice treated with i.p. injection of IL-2 pDNA:lipid on days 5-10 after tumor cell injection. The DNA was complexed at either a 1:1 (15A and 15B) or 5:1 (FIGS. 15C and 15D) DNA:DMRIE mass ratio (100 µg pDNA). Plasmid DNA without lipid was not effective (FIGS. 15E and 15F)

FIG. ^{16A and 16B} ~~16~~ shows that i.p. mIL-2 plasmid DNA (VR1110):lipid treatment inhibits tumor growth (FIG. 16A) and enhances survival (FIG. 16B) in the MOT tumor model in C3H/HeN mice. MOT tumor-bearing mice received three alternative-day i.p. injections of a complex of pDNA:DMRIE/DOPE (1:1 DNA:DMRIE mass ratio, 100 µg of plasmid DNA).

FIG. ^{17A and 17B} ~~17~~ shows a significant reduction in MOT tumor growth and increased survival of mice treated with i.p. injection of IL-2 DNA:lipid followed by debulking of tumor ascites. MOT tumor-bearing mice received six consecutive intraperitoneal injections of a complex of pDNA:DMRIE/DOPE (1:1 DNA:DMRIE mass ratio, 100 µg of pDNA) and

debulked of 5 ml of tumor ascites 4 days after the last DNA:lipid injection (n=10).

18A and 18B

FIG. 18 shows dose-response of mIL-2 pDNA (VR1110):lipid treatment in the MOT tumor model. C3H/HeN mice bearing MOT tumor were injected with 25, 50 or 100 μ g of VR1110:DMRIE/DOPE on days 5, 8 and 11 after MOT tumor cell injection. In mice treated with 50 or 100 μ g of VR1110, tumor growth was significantly reduced ($p=0.002$) and survival significantly enhanced ($p=0.01$) by day 15 post tumor cell inoculation compared to the control. Tumor-bearing mice treated with 25 μ g of VR1110:lipid were not significantly different from the control mice for either tumor volume or survival (n=15).

19A - 19P

FIG. 19 shows the cytokine profile of ovarian tumor ascites in C3H/HeN mice MOT tumor model following mIL-2 pDNA (VR1110):lipid treatment. Mice received i.p. injections of a complex of pDNA/DMRIE/DOPE (1:1 DNA/DMRIE mass ratio, 100 μ g of plasmid DNA) on days 5, 8 and 11 after tumor cell implant. Two days after each injection, mice were sacrificed (5 mice for each time point), and the ascites were collected and analyzed for cytokine concentration. The level of IL-2 (days 7, 10 and 13) as well as IFN γ and GM-CSF (days 10 and 13) were markedly elevated suggesting that IL-2 upregulates IFN γ and GM-CSF production.

20A and 20B

FIG. 20 shows that i.p. mIFN α pDNA (VR4111):lipid treatment enhances survival (FIG. 20B) in the MOT tumor model in C3H/HeN mice. MOT tumor-bearing mice received three alternative-day i.p. injections of a complex of pDNA:DMRIE/DOPE (1:1 DNA:DMRIE mass ratio, 100 μ g of plasmid DNA).

FIG. 21 shows the schematic contents of plasmid DNAs used in the examples that follow. All vectors contain a pUC19 origin of replication, human cytomegalovirus intron A, and the bacterial kanamycin resistance gene. "Lux" denotes the coding region encoding luciferase, from the firefly, *Photinus pyralis*; "CMV" denotes the human cytomegalovirus immediate

early region - promoter and enhancer; "BGH" denotes the bovine growth hormone transcriptional terminator; "LacZ" denotes the coding region encoding the β -galactosidase protein of *Escherichia coli*; "RSV" denotes the Rous sarcoma virus promoter and enhancer; "EPO" denotes the coding region encoding murine erythropoietin; "SEAP" denotes the coding region for secreted human placental alkaline phosphatase; "Rat preproinsulin" denotes the coding region for rat preproinsulin containing a point mutation to change histidine B10 (codon CAC) to aspartic acid (codon GAC), Abai, A.M., *et al. Human Gene Therapy* 10:2637-2649 (1999); "IFN-omega" denotes the coding region encoding human interferon- ω ; "mRGB" denotes the modified rabbit β -globin transcriptional terminator; and "NP" denotes the coding region encoding the nucleoprotein of influenza virus A/PR/8/34. Intermediate and parental plasmids *VR1012, **VR1255 and ***VIJ were prepared as described by Manthorpe, M. *et al., Hum. Gene Ther.* 4:419-431 (1993), Hartikka, J. *et al., Hum. Gen. Ther.* 7:1205-1217 (1996), and Montgomery, D.L. *et al., DNA Cell Biol.* 12:777-783 (1993), respectively. VR1043 was derived from VR1012 by replacing the SacI-NdeI CMV promoter enhancer fragment with the RSV promoter enhancer.

FIG. 22A is a bar graph demonstrating the effectiveness of sodium phosphate concentration on luciferase expression in mouse muscle. Fifty μ g of plasmid VR1223 DNA per 50 μ l sodium phosphate solution at the indicated molar concentrations was injected into mouse quadriceps and the muscles were extracted and assayed for enzyme activity 7 days later. Bars represent Standard Error of the Mean (n = 50, 5 experiments each with n = 10 per concentration). Peak expression occurred with DNA dissolved in 150 mM sodium phosphate, and yielded 386 ng luciferase per muscle which is 4.3-fold higher than the saline average (dashed line at 89 ng luciferase per muscle). The 80, 100, 150 and 200 mM sodium phosphate values were significantly higher than saline by Mann-Whitney rank sum test (p < 0.05).

FIG. 22B is a bar graph demonstrating the effect of pH of the sodium phosphate and potassium phosphate solutions on luciferase expression in

mouse muscle. Fifty μg of plasmid VR1223 DNA per 50 μl sodium phosphate and potassium phosphate solution at the indicated pH was injected into mouse quadriceps and the muscles were extracted and assayed for enzyme activity 7 days later. Bars represent Standard Error of the Mean ($n = 20$ muscles per group).

FIG. 22C is a graph plotting the effect of pH of the various salt solutions listed in Table 11-A on luciferase expression in mouse muscle.

FIG. 22D is a graph plotting the effect of osmolarity of the various salt solutions listed in Table 11-B on luciferase expression in mouse muscle.

FIG. 23 is a bar graph demonstrating the reproducibility of the enhancement of luciferase expression in muscle upon delivery in 150 mM sodium phosphate. In each of nine experiments, ten quadriceps muscles in 5 mice per group were injected with 50 μg of plasmid VR1223 DNA dissolved in 50 μl saline or in 150 mM sodium phosphate (NaP). Bars represent the average ng luciferase per muscle for each experiment numbered 1 through 9. Error bars represent Standard Error of the Mean.

FIG. 24 shows the comparison of the effect of a 150 mM sodium phosphate solution on the expression of three reporter genes. Fifty μg of plasmid VR1223 (luciferase), 10 μg of plasmid VR1418 (β -galactosidase, or LacZ) or 50 μg of plasmid VR4151 (human IFN ω) dissolved in 50 μl saline or in 150 mM sodium phosphate solution were injected into the quadriceps muscles of BALB/c mice. For luciferase and LacZ DNAs, the muscles were extracted and assayed 7 days later for enzyme activity. For IFN- ω DNA, serum was collected at 7 days after the injection and assayed for IFN- ω protein. Values are expressed as average ng of gene product per muscle or per ml serum. Bars represent Standard Error of the Mean. For luciferase, $n_{\text{Saline}} = 413$, $n_{\text{NaP}} = 120$; for β -galactosidase, $n_{\text{Saline}} = 119$, $n_{\text{NaP}} = 180$; for IFN- ω , $n_{\text{Saline}} = 10$, $n_{\text{NaP}} = 9$. The average expression in NaP was significantly higher than saline by Mann-Whitney rank sum test for luciferase ($p = 0.001$), β -galactosidase ($p = 0.001$) and IFN- ω ($p = 0.02$).

FIG. 25 shows long-term effects of a 150 mM sodium phosphate solution on the expression of secreted reporter gene products. Compositions comprising plasmids VR3301 encoding human placental alkaline phosphatase (SEAP), VR3502 encoding rat preproinsulin, and VR2901 encoding mouse erythropoietin, dissolved in saline or in 150 mM sodium phosphate, were injected bilaterally into mice as described in Example 1. At the indicated times after injections, serum was collected and assayed for SEAP or proinsulin expression, or hematocrits were measured as an indication of erythropoietin expression. Control mice injected with plasmid DNA encoding canine clotting Factor IX (open triangles in the lower graph) in 150 mM sodium phosphate exhibited an average hematocrit of 46. Bars represent Standard Error of the Mean ($n = 10$). By the Mann-Whitney rank sum test, the sodium phosphate values were significantly different (p values all < 0.007) from the saline values for each time point and for all three reporters.

FIG. 26 shows the effect of a 150 mM sodium phosphate solution on DNA degradation in mouse muscle extract or serum. VR1255 plasmid DNA dissolved in each of 4 aqueous solutions was spiked with 10% (v/v) unbuffered mouse Muscle Extract or Serum and the spiked solutions were incubated for 2 hours at 37°C . The reactions were neutralized with SDS + EDTA and analyzed by agarose gel electrophoresis. The top row of four lanes are from the solutions spiked with muscle extract and the bottom row of lanes are from the solutions spiked with serum. The DNA samples from left to right are in: Control solution (pre-neutralized sample in water), Water, Saline, or NaP (150 mM sodium phosphate). On the right side of the right lane are indicated the position of the bands corresponding to nicked, linear, closed circular, and degraded plasmid DNA. The numbers at the bottom of the lower lanes are 7 day luciferase expression values taken from Tables 1 and 2 where DNA in the indicated vehicle was injected into muscle.

FIG. 27 shows the effects of a 150 mM sodium phosphate solution on DNA vaccination. Mice were vaccinated bilaterally in the quadriceps muscle with 5 μg of plasmid VR4700, encoding the influenza virus nucleoprotein, which was dissolved in 50 μl of saline or in 50 μl of 150 mM sodium

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phosphate on days 0 and 21. (A) Serum was collected at day 42 and assayed for anti-NP antibody titer by ELISA. Three separate experiments were performed with $n = 10$ mice each, labeled 1-3. The average (Avg.) of all three experiments is indicated in the black bar. Values are expressed as anti-NP specific titer ($n = 10$, 2 experiments with $n = 5$). Error bars represent Standard Error of the Mean. Average anti-NP titers from NaP groups 1-3 were significantly different from the saline averages by Mann-Whitney rank sum test ($p < 0.04$) as was the average titers from all 3 groups ($p < 0.001$). (B) At day 60 the spleens were collected, dissociated and assayed for the presence of NP-specific cytolytic T lymphocyte activity. Splenocytes from unvaccinated mice served as controls ("Naïve"). Average % NP specific lysis from the saline and NaP groups were not significantly different by Mann Whitney rank sum test.

FIG. 28 shows the effects of sodium phosphate solutions on luciferase expression in lung following delivery of compositions comprising plasmid DNA encoding luciferase. Mouse lungs were intranasally instilled with compositions comprising $132 \mu\text{g}$ of plasmid VR1223 encoding luciferase, complexed with GAP-DLRIE/DOPE (1:1) cationic liposomes at a molar ratio of 4:1 DNA to lipid in water or in various aqueous solutions of sodium phosphate. The lungs were extracted 3 days later and assayed for luciferase activity. Values are expressed in ng luciferase per lung \pm Standard Error of the Mean (n_{water} and $n_{2.5\text{mMNaP}} = 35$; $n_{10\text{mMNaP}}$, and $n_{150\text{mMNaP}} = 15$ with $n = 5$ per each individual experiment). The 2.5 mM NaP solution averages were significantly different by Mann-Whitney rank sum test from all the other groups ($p = <0.001$).

Detailed Description of the Preferred Embodiments

The present invention is broadly directed to treatment of cancer by administering *in vivo*, into a tissue of a mammal suffering from cancer, at least one polynucleotide construct comprising at least one polynucleotide encoding at least one cytokine, or at least one active fragment thereof. The polynucleotide construct is incorporated into the cells of the mammal *in vivo*, and a therapeutically effective amount of a cytokine is produced *in vivo*, and delivered to tumor cells. Combinations of cytokine-encoding polynucleotides can be administered.

The present invention provides a pharmaceutical composition comprising about 1 ng to 20 mg of a non-infectious, non-integrating polynucleotide construct comprising a polynucleotide selected from the group consisting of (a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; (b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, and wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and (c) a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. The pharmaceutical composition can be used to practice all of the methods of the present invention.

The present invention also provides a pharmaceutical composition obtained by complexing a polynucleotide selected from the group consisting of (a) a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the

polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; (b) a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and (c) a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, with one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

The pharmaceutical composition of the present invention can be a polynucleotide construct comprising a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the pharmaceutical composition of the present invention can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the pharmaceutical composition of the present invention can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells

in vitro; and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof.

The pharmaceutical composition of the present invention comprises at least one polynucleotide construct comprising at least one polynucleotide encoding an IFN ω , or an active fragment thereof. Preferably, the polynucleotide construct contains a polynucleotide encoding a human IFN ω . More preferably, IFN ω is encoded by nucleotides 1 to 585 in SEQ ID No. 7 (corresponding to amino acids -23 to 172 in SEQ ID No. 8), or by nucleotides 70 to 585 in SEQ ID No. 7 (corresponding to amino acids 1 to 172 in SEQ ID No. 8). Most preferably, the polynucleotide construct is VR4151 in SEQ ID No. 4. The polynucleotide construct may be complexed with one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Preferably, the polynucleotide construct is complexed with one or more cationic lipids. More preferably, the polynucleotide construct is complexed with one or more cationic lipids and one or more neutral lipids. Still more preferably, the cationic lipid is (\pm)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide (DMRIE) and the neutral lipid is 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) such that the mass ratio of polynucleotide construct to lipid is from about 10:1 and about 0.5:1. More preferably, the mass ratio of polynucleotide construct to lipid is from about 5:1 and about 1:1. Still more preferably, the mass ratio of polynucleotide construct to lipid is about 5:1.

Cytokine-encoding plasmids discussed herein include VR4102 (hIFN α in the VR1012 vector) (SEQ ID No. 1), VR4112 (hIFN α in the VR1055 vector) (SEQ ID No. 2), VR4150 (hIFN ω in the VR1012 vector) (SEQ ID No. 3), VR4151 (hIFN ω in the VR1055 vector) (SEQ ID No. 4), VR4101 (mIFN α in the VR1012 vector) (SEQ ID No. 5), VR4111 (mIFN α in the VR1055 vector) (SEQ ID No. 6), and VR1110 (mIL-2 in the VR1012 vector), VR1103

(hIL-2 in the VR1012 vector) (SEQ ID No: 25), VR4001 (mIL-12 in the VR1033 vector), and VR1700 (mGM-CSF in the VR1012 vector).

Cytokine-encoding cDNAs discussed herein include the cDNA for hIFN ω (SEQ ID No. 7), the cDNA for hIFN α (SEQ ID No. 9), the cDNA for mIFN α (SEQ ID No. 11), the cDNA for hIL-2 (SEQ ID No. 13 and the coding portion of SEQ ID No. 25), the cDNA for mIL-2 (for example, as disclosed in Kashima *et al.*, *Nature* 313:402-404 (1985), which is hereby incorporated by reference) the cDNA for mIL-12 (for example, as disclosed in Tone *et al.*, *Eur. J. Immunol.* 26:1222-1227(1996), which is hereby incorporated by reference), and the cDNA for mGM-CSF (for example, as disclosed in Gough *et al.*, *EMBO J.* 4:645-653 (1985), which is hereby incorporated by reference). Cytokine polypeptides discussed herein include hIFN ω (SEQ ID No. 8), hIFN α (SEQ ID No. 10), mIFN α (SEQ ID No. 12), and hIL-2 (SEQ ID No. 14 and SEQ ID No. 26).

By "stringent conditions" is intended a hybridization by overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (750 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μ g/ml denatured, sheared salmon sperm DNA, followed by repeatedly washing the filters (at least three times) in 0.1x SSC and 0.1% sodium dodecyl sulfate (w/v) for 20 minutes at about 65°C.

By "active fragment" is intended a fragment of a cytokine that displays the antiproliferative activity of the mature or full length cytokine. For example, a full length hIFN ω is set forth in amino acids -23 to 172 of SEQ ID No. 8. The corresponding mature hIFN ω is set forth in amino acids 1 to 172 of SEQ ID No. 8. Active fragments of hIFN ω include, but are not limited to a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, a polypeptide comprising amino acids 61-172 in SEQ ID No. 8, a polypeptide comprising amino acids 41-172 in SEQ ID No. 8, and a polypeptide comprising amino acids 21-172 in SEQ ID No. 8. A full length hIFN α is set forth in amino acids -23 to 166 of SEQ ID No. 10. The corresponding mature hIFN α is set forth in

amino acids 1 to 166 of SEQ ID No. 10. Active fragments of hIFN α include, but are not limited to a polypeptide comprising amino acids 83-166 in SEQ ID No. 10, a polypeptide comprising amino acids 61-166 in SEQ ID No. 10, a polypeptide comprising amino acids 41-166 in SEQ ID No. 10, and a polypeptide comprising amino acids 21-166 in SEQ ID No. 10. Full length hIL-2 is set forth in amino acids -20 to 133 of SEQ ID No. 14. The corresponding mature hIL-2 is set forth in amino acids 1 to 133 of SEQ ID No. 14. Active fragments of hIL-2 include, but are not limited to a polypeptide comprising amino acids 58 to 105 in SEQ ID No. 14, and a polypeptide comprising amino acids 20 to 126 in SEQ ID No. 14.

Assays of antiproliferative activity *in vitro* are well known to those of ordinary skill in the art. For example, one antiproliferation assay that can be used is to treat cultured cells, such as human ovarian NIH-OVCAR3 cells (ATCC, Rockville, MD), with supernatants from human melanoma UM449 cells transfected with the polynucleotide construct containing a polynucleotide encoding an IFN ω or an active fragment thereof. In this antiproliferation assay, NIH-OVCAR3 cells are cultured and plated in 96 well-tissue culture plates. The plates are incubated for 24 hours at 37°C in a humidified 5% CO₂ atmosphere. Twenty μ l of tissue culture supernatants from transfected UM449 cells are added to duplicate wells. An interferon reference standard (e.g., human leukocyte interferon, Sigma Chemical Co., St. Louis, MO) is included in each assay. The cells are incubated with the test samples or the interferon standard for an additional 72 hours at 37°C. To quantitate the effects on cell proliferation, 50 μ l of XTT/ECR substrate (Cell Proliferation Kit, Boehringer Mannheim, Indianapolis, IN) is added to each well and the plates are incubated for an additional 24 hours at 37°C prior to measurement of the OD₄₉₀. Other cell lines can be used in the antiproliferation assay. For example, any of the cells listed on Table 1 can be used. Another antiproliferation assay that can be used is provided in Nieroda, *et al* (*Mol. Cell. Differentiation* 4: 335-351 (1996)).

For treatment of cancer, a polynucleotide construct comprising a polynucleotide encoding a cytokine can be delivered locally, systemically or

intra-cavity. In the "systemic delivery" embodiment of the invention, one or more polynucleotide construct comprising one or more polynucleotide encoding one or more cytokine is administered into a tissue such that the polynucleotide is expressed as the cytokine *in vivo* and the cytokine is released into the circulation, and such that a therapeutically effective amount of the cytokine is systemically delivered to the tumor. In this embodiment, the polynucleotide construct can be administered within *ex vivo* cells or associated with *ex vivo* cellular material. Preferably, the cytokine is an IFN ω , IFN α , IFN τ , IFN γ , IFN β , IL-1, IL-2, IL-4, IL-7, IL-12, IL-15, IL-18, GM-CSF, or any combination of these, or any combination of one or more of these and one or more additional cytokines. More preferably, the cytokine is an IFN α , IFN ω , IL-2, or IL-12. Most preferably, the cytokine is an IFN α or IFN ω . Examples of the combination are a polynucleotide encoding an IFN ω and an IFN α ; a polynucleotide encoding an IFN ω and an IL-2; a polynucleotide encoding an IFN α and an IL-2; and a polynucleotide encoding an IFN ω , an IFN α , and an IL-2. More preferably, the polynucleotide construct contains a polynucleotide encoding an IFN ω and/or an IFN α . Even more preferably, the polynucleotide construct contains a polynucleotide encoding a human IFN ω and/or a human IFN α . Even more preferably, the polynucleotide encodes a human IFN ω . Preferably, the polynucleotide construct is administered free from *ex vivo* cells and free from *ex vivo* cellular material.

In this embodiment, administration can be into tissue including but not limited to muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph nodes, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, or connective tissue. Preferably, the administration is into muscle tissue, *i.e.*, skeletal muscle, smooth muscle, or myocardium, and the polynucleotide construct is naked. Most preferably, the muscle is skeletal muscle. For polynucleotide constructs in which the polynucleotide encoding a cytokine is DNA, the DNA can be operably linked to a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells.

By "naked" is meant that the polynucleotide construct is free from association with any delivery vehicle known in the art that can act to facilitate entry into cells, for example, from transfection-facilitating proteins, viral particles, liposomes, cationic lipids, and calcium phosphate precipitating agents.

As used herein, "*ex vivo*" cells are cells into which the polynucleotide construct is introduced, for example, by transfection, lipofection, electroporation, bombardment, or microinjection. The cells containing the polynucleotide construct are then administered *in vivo* into mammalian tissue. Such *ex vivo* polynucleotide constructs are well-known to those of ordinary skill in the art. For example, see Beldegrun, A., *et al.*, *J. Natl. Cancer Inst.* 85: 207-216 (1993); Ferrantini, M. *et al.*, *Cancer Research* 53: 1107-1112 (1993); Ferrantini, M. *et al.*, *J. Immunology* 153: 4604-4615 (1994); Kaido, T., *et al.*, *Int. J. Cancer* 60: 221-229 (1995); Ogura, H., *et al.*, *Cancer Research* 50: 5102-5106 (1990); Santodonato, L., *et al.*, *Human Gene Therapy* 7:1-10 (1996); Santodonato, L., *et al.*, *Gene Therapy* 4:1246-1255 (1997); and Zhang, J.-F. *et al.*, *Cancer Gene Therapy* 3: 31-38 (1996).

The polynucleotide construct is administered in a "cell-free" fashion when it is administered independently, *i.e.*, free of *ex vivo* cells or *ex vivo* cellular material.

In the "local cytokine delivery" embodiment of the present invention, a polynucleotide construct comprising a polynucleotide encoding IFN ω and/or IFN α is administered *in vivo* into or near a tumor of a mammal, such that the polynucleotide is incorporated into the cells of the tumor. Tumor cells subsequently express the interferon polypeptide in an amount effective to treat cancer.

In this embodiment, a polynucleotide construct comprising a polynucleotide encoding an IFN ω and/or an IFN α can be administered into the tumor. Alternatively, the polynucleotide construct can be administered into non-tumor cells surrounding a tumor, near a tumor, or adjacent to a tumor, such that a therapeutically effective amount of an IFN ω and/or an IFN α is produced *in vivo* near or within the tumor and is delivered to the malignant

cells of the tumor. One way to provide local delivery of the polynucleotide construct is by administering intravenously a polynucleotide construct comprising a tumor-targeted promoter, wherein the polynucleotide is incorporated into the cells of the tumor and the cytokine is expressed in the tumor in an amount effective to treat cancer. Preferably, the polynucleotide construct is administered into the tumor.

In the "intra-cavity delivery" embodiment, the present invention provides a method of selectively transfecting malignant cells in a tumor-bearing body cavity of a mammal by introducing a polynucleotide construct into the body cavity, wherein the polynucleotide is incorporated into tumor cells and the tumor cells subsequently express the protein encoded by the polynucleotide in an amount effective to treat cancer. The polynucleotide construct is administered free from *ex vivo* cells and free from *ex vivo* cellular material.

A cavity is a space within the body that can confine a fluid volume for some period of time. The cavity can either be present in a normal animal, or it can be produced as a result of disease, surgery or trauma. Cavities in the normal animal include the peritoneum, the cerebrospinal fluid space, the ventricles of the brain, the plural space around lung, the bronchiolar airways, the nasal sinus, the bladder, the vagina, the ear, the synovium of various joints (knee, hip etc.), the internal network of salivary gland tissue, and the gastrointestinal tract including stomach. Surgical removal of tumor tissue can also produce a space which fits the definition of a cavity. An open wound produced by trauma or surgery and closed by suture can be defined as a cavity, and the area under a blister produced by an infection, abrasion or a burn also fits the definition.

There are special bioavailability considerations when a gene delivery system is administered into a cavity. First the fluid volume in the cavity can be substantially comprised of the vehicle in which the delivery system is suspended. Second, the delivery system can have particular access to cells that are either suspended in the cavity, or that are lining the surface of the cavity. Third, in some cases normally differentiated cells that are lining the cavity

may be embedded in an extracellular matrix and, may not be accessible to the delivery system. Thus, the delivery system may preferentially transfect cells that are growing outside the normal extracellular matrix and avoid the cells that are growing within the extracellular matrix, conferring a kind of cell selectivity to the delivery system.

With respect to the first point, body fluids such as serum, have been shown to inhibit gene delivery systems. For example, the transfection activities of Lipofectin and LipofectAMINE are inhibited by serum. It is thought that serum factors bind to cationic lipid/DNA complexes and block their uptake into cells. In cavity models the endogenous fluid volume can be removed, the cavity can be washed, and the delivery system can be administered into the cavity in a vehicle that is compatible with optimal gene delivery efficacy. Thus the cavity model allows the investigator to create a fluid environment which allows for optimal gene delivery potency.

With respect to the second point, cells that are either floating in the cavity or are lining the surface of the cavity have preferential access to the delivery system and can be preferentially transfected relative to other cells in the body. Since the delivery system is confined within the cavity, peripheral cells in the body outside of the cavity will not be transfected. Thus, there is tissue targeting to the cells within the cavity. For example, gene delivery systems administered into the peritoneal cavity will have access to metastatic tumor cells derived from colon or ovarian cancers that are floating in the peritoneum or are attached to the surfaces of the peritoneum. Delivery systems administered into the plural space should transfect cancer cells in the plural effusion. Delivery systems administered into the cerebral spinal fluid should have access to metastatic cancer cells present there.

With respect to the third point, differentiated cells that are present in normal tissues are often embedded into an extracellular matrix. This matrix can be difficult to penetrate with large particulate delivery systems. Some cells, such as poorly differentiated tumor cells, that are present in cavities can grow outside of the normal extracellular matrix and are therefore more accessible to gene delivery systems. In this way the delivery system can

preferentially transfect those cells that are growing outside of the extracellular matrix and not transfect those cells that are growing within the extracellular matrix. This is another form of in vivo, cell type specific targeting. Examples of normal cells that are not embedded in an extracellular matrix and are therefore more accessible to gene delivery systems are, bronchial airway cells, lung cells in the plural space, and endipinal cells lining the surface of the ventricles of the brain. Normal bladder cells that line the surface of the bladder are embedded in a tight extracellular matrix and are therefore not readily accessible to a gene delivery system delivered into the bladder, but tumor cells which grow up and out of the extracellular matrix into the bladder vesicle are accessible to gene delivery systems administered into the bladder vesicle. Thus normal bladder tissue would be expected to resist transfection whereas, bladder tumor would be expected to be transfectable.

A preferred application of the intra-cavity delivery embodiment is in the treatment of peritoneally disseminated cancers. More specifically, a mammal bearing peritoneal tumor may be injected i.p. with an effective amount of a polynucleotide complexed with a lipid in a physiologically acceptable diluent in a total volume sufficient to access the entire body cavity. The mammal may have tumor ascites in the peritoneal cavity as in an ovarian cancer. In the most preferred application, this methodology may be used in treating ovarian cancer of a human.

Debulking of tumor ascites is commonly performed on human ovarian cancer patients. Debulking involves removal of tumor ascites from the peritoneal cavity. In humans bearing ovarian tumor ascites, the ascites fluid would be debulked by insertion of a catheter i.p. followed by periodic draining of ascites fluid. It is contemplated that the tumor ascites would be debulked before and/or after the i.p. administration of the polynucleotide formulation of the present invention.

Transfection efficacy of the intra-cavity delivery embodiment may be determined by collecting the tumor ascites and serum at various times after the injection and performing diagnostic assays appropriate for the encoded

molecule(s). Naturally, other means of determining tumor mass, growth, and viability may also be used to assess the effectiveness of the present invention.

Preferred polynucleotides for the intra-cavity delivery embodiment may encode not only immunogenic molecules such as cytokines (*e.g.*, interleukins 1-18 and $\alpha/\beta/\gamma/\omega$ -interferons, colony stimulating factors, *e.g.*, G-CSF, GM-CSF, M-CSF, and tumor necrosis factors), but also chemokines (*e.g.*, C-X-C and C-C), Class I and II histocompatibility antigens, costimulatory molecules (*e.g.*, B7-1, B7-2, CAMs, and flt3 ligand), growth factors (*e.g.*, epidermal growth factors, fibroblast growth factors, transforming growth factors and growth hormone), and the like. The polynucleotide may also encode bacterial antigens, viral glycoproteins, enzymes (*e.g.*, lysozymes), recombinant antibodies, molecules that interfere with cellular adhesion, adhesion molecules, proliferation and vascular inhibitory factors, ribozymes, and antisense RNAs targeted toward key oncogenic or tumor growth proteins. Moreover, selective delivery of toxic peptides (*e.g.*, ricin, diphtheria toxin, or cobra venom factor) or proteins capable of synthesizing toxic compounds (*e.g.* thymidine kinase and cytosine deaminase) to the malignant cells may have therapeutic benefits. The polynucleotide may also comprise a tumor suppressor gene (*e.g.*, p53). Preferred polynucleotides encode cytokines. Preferred cytokines are IL-2, IFN ω , and IFN α . IL-2 is most preferred.

For treatment of cancer by any of the above disclosed embodiments, any polynucleotide encoding an IFN ω , or an active fragment thereof, can be used. For example, the polynucleotide construct can be a construct comprising a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 7 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the construct can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions,

deletions, or insertions, is identical to amino acids -23 to 172 or 1 to 172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the construct can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising amino acids 86-172 in SEQ ID No. 8, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Preferably, IFN ω is encoded by nucleotides 1 to 585 in SEQ ID No. 7 (corresponding to amino acids -23 to 172 in SEQ ID No. 8), or by nucleotides 70 to 585 in SEQ ID No. 7 (corresponding to amino acids 1 to 172 in SEQ ID No. 8). More preferably, the polynucleotide construct is VR4151.

For treatment of cancer, any polynucleotide encoding IFN α , or active fragment thereof, can also be used. For example, the polynucleotide construct can be a construct comprising a polynucleotide that hybridizes under stringent conditions to the nucleotide sequence of SEQ ID No. 9 or the complement thereof, wherein the polynucleotide sequence encodes a polypeptide that has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the construct can be a polynucleotide construct comprising a polynucleotide that encodes a polypeptide comprising an amino acid sequence which, except for at least one but not more than 20 amino acid substitutions, deletions, or insertions, is identical to amino acids -23 to 166 or 1 to 166 in SEQ ID No. 10, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*, and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Alternatively, the construct can be a polynucleotide construct comprising a

polynucleotide that encodes a polypeptide comprising amino acids 83-166 in SEQ ID No. 10, wherein the polypeptide has antiproliferative activity when added to NIH-OVCAR3 cells *in vitro*; and one or more cationic compounds selected from the group consisting of cationic lipids, cationic peptides, cationic proteins, cationic polymers, and mixtures thereof. Preferably, IFN α is encoded by nucleotides 1 to 567 in SEQ ID No. 9 (corresponding to amino acids -23 to 166 in SEQ ID No. 10), or by nucleotides 1 to 567 in SEQ ID No. 9 (corresponding to amino acids 1 to 166 in SEQ ID No. 10). Preferably, the polynucleotide construct is VR4112.

For polynucleotide constructs that do not contain a polynucleotide encoding IFN ω , the polynucleotide construct is preferably a cell-free construct. For polynucleotide constructs that contain a polynucleotide encoding IFN ω , the polynucleotide construct can be administered either within *ex vivo* cells or free of *ex vivo* cells or *ex vivo* cellular material. Preferably, the polynucleotide construct is administered free of *ex vivo* cells or *ex vivo* cellular material.

In the "local delivery" and "intra-cavity delivery" embodiments, the polynucleotide construct is preferably complexed with one or more cationic compounds. More preferably, the polynucleotide construct is complexed with one or more cationic lipids by ionic interaction. Generally, the complex then contacts the cell membrane and is transfected into the cell. This transfection mechanism is referred to as "lipofection," and is a highly efficient transfection procedure (Felgner, *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1987); and Felgner, *et al.*, *Nature* 337:387-388, 1989). Still more preferably, the polynucleotide construct is complexed with one or more cationic lipids and one or more neutral lipids.

For purposes of the present invention, lipid refers to a synthetic or naturally occurring compound that possesses both a lipophilic region and a polar region, commonly referred to as a head group. Preferred cationic compounds are cationic lipids. Cationic lipids are described in U.S. Pat. Nos. 4,897,355; 4,946,787; 5,049,386; 5,264,618; 5,279,833; 5,334,761; 5,429,127; 5,459,127; 5,589,466; 5,676,954; 5,693,622; 5,580,859; 5,703,055;

and 5,578,475; and international publications WO 04/9469, WO 95/14381, 95/14651, 95/17373, 96/18372, 96/26179, 96/40962, 96/40963, 96/41873, and 97/00241, and documents cited therein. As illustrated in the above-cited patents and patent applications, cationic lipids comprise structural features that may be present in a variety of core molecular classes.

Examples of cationic lipids are 5-carboxyspermylglycine dioctadecylamide (DOGS) and dipalmitoyl-phosphatidylethanolamine-5-carboxyspermylamide (DPPEs). Cationic cholesterol derivatives are also useful, including {3 β -[N-N',N'-dimethylamino)ethane]-carbomoyl}-cholesterol (DC-Chol). Dimethyldioctadecyl-ammonium bromide (DDAB), N-(3-aminopropyl)-N,N-(bis-(2-tetradecyloxyethyl))-N-methyl-ammonium bromide (PA-DEMO), N-(3-aminopropyl)-N,N-(bis-(2-dodecyloxyethyl))-N-methyl-ammonium bromide (PA-DELO), N,N,N-tris-(2-dodecyloxy)ethyl-N-(3-amino)propyl-ammonium bromide (PA-TELO), and N¹-(3-aminopropyl)((2-dodecyloxy)ethyl)-N²-(2-dodecyloxy)ethyl-1-piperazinaminium bromide (GA-LOE-BP) can also be employed in the present invention.

Non-diether cationic lipids, such as DL-1,2-dioleoyl-3-dimethylaminopropyl- β -hydroxyethylammonium (DORI diester), 1-O-oleyl-2-oleoyl-3-dimethylaminopropyl- β -hydroxyethylammonium (DORI ester/ether), and their salts promote *in vivo* gene delivery. Preferred cationic lipids comprise groups attached via a heteroatom attached to the quaternary ammonium moiety in the head group. A glyceryl spacer can connect the linker to the hydroxyl group.

Preferred cationic lipids are 3,5-(N,N-dilysyl)-diaminobenzoyl-3-(DL-1,2-dioleoyl-dimethylaminopropyl- β -hydroxyethylamine) (DLYS-DABA-DORI diester), 3,5-(N,N-di-lsyl)amino-benzoylglyceryl-3-(DL-1,2-dioleoyl-dimethylaminopropyl- β -hydroxyethylamine) (DLYS-DABA-GLY-DORI diester), and (\pm)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide (DMRIE).

Also preferred are (\pm)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride (DOSPA), (\pm)-N-(2-aminoethyl)-N,N-dimethyl-2,3-

bis(tetradecyloxy)-1-propaniminium bromide (β -aminoethyl-DMRIE or β AE-DMRIE) (Wheeler, *et al.*, *Biochim. Biophys. Acta* 1280:1-11 (1996)), and (\pm)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propaniminium bromide (GAP-DLRIE) (Wheeler, *et al.*, *Proc. Natl. Acad. Sci. USA* 93:11454-11459 (1996)), which have been developed from DMRIE.

Other examples of DMRIE-derived cationic lipids that are useful for the present invention are (\pm)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(bis-dodecyloxy)-1-propanaminium bromide (GAP-DDRIE), (\pm)-N-(3-aminopropyl)-N,N-dimethyl-2,3-(bis-tetradecyloxy)-1-propanaminium bromide (GAP-DMRIE), (\pm)-N-((N"-methyl)-N'-ureyl)propyl-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (GMU-DMRIE), (\pm)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide (DLRIE), and (\pm)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis-([Z]-9-octadecenyl-oxy)propyl-1-propaniminium bromide (HP-DORIE).

The lipids of the lipid-containing formulation can comprise a cationic lipid alone, or further comprise a neutral lipid such as cardiolipin, phosphatidylcholine, phosphatidylethanolamine, dioleoylphosphatidylcholine, dioleoylphosphatidyl-ethanolamine, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), sphingomyelin, and mono-, di- or tri-acylglycerol. Other additives, such as cholesterol, fatty acid, ganglioside, glycolipid, neobee, niosome, prostaglandin, sphingolipid, and any other natural or synthetic amphiphiles, can also be used. A preferred molar ratio of cationic lipid to neutral lipid in these lipid-containing formulations is from about 9:1 to about 1:9; an equimolar ratio is particularly preferred. The lipid-containing formulation can further comprise a lyso lipid (*e.g.*, lyso-phosphatidylcholine, lysophosphatidyl-ethanolamine, or a lyso form of a cationic lipid).

More preferably, the cationic lipid is (\pm)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propaniminium bromide (DMRIE) and the neutral lipid is 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) such that the mass ratio of polynucleotide construct to lipid is from about 10:1 and about 0.5:1. Still more preferably, the mass ratio of polynucleotide

construct to lipid is from about 5:1 and about 1:1. Still more preferably, the mass ratio of polynucleotide construct to lipid is about 5:1.

Lipid-containing pharmaceutical composition for use in a complex with the polynucleotide construct of the present invention can also comprise cationic lipid together with an effective amount of a lysophosphatide. The lysophosphatide can have a neutral or a negative head group. Lysophosphatidylcholine and lysophosphatidyl-ethanolamine are preferred, and 1-oleoyl lysophosphatidylcholine is particularly preferred. Lysophosphatide lipids are advantageously present in the lipid-containing formulation in a 1:2 ratio of lysolipid to cationic lipid. Lyso forms of a cationic lipid can also be used to promote polynucleotide delivery. These lyso forms are advantageously present in effective amounts up to about one-third of the total cationic lipid in the lipid-containing formulations.

In a formulation for preparing DNA: lipid complexes, the cationic lipid can be present at a concentration of between about 0.1 mole % and about 100 mole %, preferably about 5 mole % and 100 mole %, and most preferably between about 20 mole % and 100 mole %, relative to other formulation components present in the formulation. The neutral lipid can be present in a concentration of between zero and about 99.9 mole %, preferably zero and about 95 mole %, and most preferably zero and about 80 mole %. In order to produce lipid vesicles having a net positive charge, the quantity of the positively charged component must exceed that of the negatively charged component. The negatively charged lipid can be present at between zero and about 49 mole %, and preferably between zero and about 40 mole %. Cholesterol or a similar sterol can be present at between zero to about 80 mole %, and preferably zero and about 50 mole %.

The polynucleotide to be delivered can be solubilized in a buffer prior to mixing with lipid vesicles. Suitable buffers include, for example, phosphate buffered saline (PBS), normal saline, Tris buffer, and sodium phosphate vehicle (100-150 mM preferred). Insoluble polynucleotides can be solubilized in a weak acid or base, and then diluted to the desired volume with a neutral buffer such as PBS. The pH of the buffer is suitably adjusted, and moreover, a

pharmaceutically acceptable additive can be used in the buffer to provide an appropriate osmolarity within the lipid vesicle.

A lipid solution comprising at least one amphipathic lipid can spontaneously assemble to form primary lipid vesicles, heterogeneous in size. Therefore, according to a preferred method, the lipids of the lipid-containing formulation, comprising at least one cationic lipid, are prepared by dissolution in a solvent such as chloroform and the mixture is evaporated to dryness as a film on the inner surface of a glass vessel. On suspension in an aqueous solvent, the amphipathic lipid molecules assemble themselves into primary lipid vesicles. These primary lipid vesicles may be reduced to a selected mean diameter by means of a freeze-thaw procedure. Vesicles of uniform size can be formed prior to drug delivery according to methods for vesicle production known to those in the art; for example, the sonication of a lipid solution as described by Felgner, *et al* (*Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987)) and U.S. Pat. No. 5,264,618.

The term "mammal" is intended to encompass a singular "mammal" and plural "mammals," and includes, but is not limited to humans; primate mammals such as apes, monkeys, orangutans, and chimpanzees; canine mammals such as dogs and wolves; feline mammals such as cats, lions, and tigers; equine mammals such as horses, donkeys, deer, zebra, and giraffe; and bears. Preferably, the mammal is a human subject.

Tumor cell formation and growth, also known as "transformation," describes the formation and proliferation of cells that have lost their ability to control cellular division; that is the cells are cancerous. "Malignant cells" are defined as cells that have lost the ability to control the cell division cycle, leading to a transformed or cancerous phenotype.

The term "non-tumor tissue" is intended to include, but is not limited to non-tumor bearing tissues such as muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, or connective tissue. Preferably, the non-tumor tissue is muscle.

Preferably, the polynucleotide construct is delivered to the interstitial space of a tumor or of non-tumor tissues. "Interstitial space" comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels.

The pharmaceutical composition and methods of the present invention can be used to treat a variety of mammalian cancers or tumors. Types of mammalian cancers and tumors that can be treated using the pharmaceutical composition and methods of the present invention include, but are not limited to all solid tumors, cutaneous tumors, melanoma, malignant melanoma, renal cell carcinoma, colorectal carcinoma, colon cancer, hepatic metastases of advanced colorectal carcinoma, lymphomas (including glandular lymphoma), malignant lymphoma, Kaposi's sarcoma, prostate cancer, kidney cancer, ovarian cancer, lung cancer, head and neck cancer, pancreatic cancer, mesenteric cancer, gastric cancer, rectal cancer, stomach cancer, bladder cancer, leukemia (including hairy cell leukemia and chronic myelogenous leukemia), breast cancer, non-melanoma skin cancer (including squamous cell carcinoma and basal cell carcinoma), hemangioma multiple myeloma, and glioma. Preferably, the cancer is melanoma, ovarian cancer, or metastases thereof.

By "treatment" is meant reduction in tumor size, a reduction in the rate of metastasis, and/or a slowing of tumor growth, and/or no worsening in disease over a specified period of time.

A systemic delivery embodiment can be particularly useful for treating nonlocalized tumors (*i.e.*, leukemia and metastases of a variety of tumors), or a disease category that might be responsive to continuous exposure by the systemic route (*i.e.*, myeloma, chronic myelogenous leukemia, lymphoma). A local delivery embodiment can be particularly useful for treating one disease condition that might be responsive to high local concentration (*i.e.*, renal cell carcinoma, melanoma). For tumors involving body cavity of a mammal,

“intra-cavity” embodiment is preferred. In particular, the use of this methodology is envisioned in treating cancers involving (i) the peritoneal cavity--pancreatic cancer, gastric cancer, ovarian cancer, mesenteric cancer, glandular lymphoma and metastatic melanoma; (ii) the thoracic cavity--lung cancer and glandular lymphoma; (iii) the rectal cavity--rectal cancer; (iv) the stomach cavity--stomach cancer; and (v) the urinary bladder vesicle--bladder cancer. When advantageous, systemic, local, and/or intra-cavity delivery can be combined, especially in a mammal having a primary site of tumor and one or more metastases.

An additional embodiment of the present invention is directed to combining any of the methods of the present invention with one or more additional cancer therapies including, but not limited to bone marrow transplant, cord blood cell transplant, surgery, chemotherapy, radiation therapy, and immunotherapy. The polynucleotide construct or pharmaceutical composition of the present invention can be administered prior to the commencement of one or more of the additional cancer therapies, during the practice of one or more of the additional cancer therapies, and after the end of one or more of the additional cancer therapies.

Types of bone marrow transplant include, but are not limited to autologous bone marrow transplant and heterologous (*i.e.*, from a donor) bone marrow transplant.

Types of surgery include, but are not limited to surgery for breast cancer, prostate cancer, colon cancer, brain cancer, and head and neck cancer.

Chemotherapeutic agents include, but are not limited to alkylating agents, including mechlorethamine, cyclophosphamide, ifosfamide, melphalan, chlorambucil, dicarbazine, streptazocine, carmustine, lomustine, semustine, chlorozotocin, busulfan, triethylenemelamine, thiotepa, hexamethylmelamine; antimetabolites, including methotrexate; pyrimidine analogs, including fluorouracil, 5-fluorouracil, floxuridine (5'-fluoro-2'-deoxyuridine), idoxuridine, cytarabine, -phosphonoacetyl-L-aspartate, 5-azacytidine, azaribine, 6-azauridine, pyrazofuran, 3-deazauridine, acivicin; purine analogs, including thioguanine, mercaptopurine, azathioprine,

pentostatin, erythrohydroxynonyladenine; vinca alkaloids, including vincristine and vinblastine; epipodophyllotoxins, including etoposide and teniposide; antibiotics, including dactinomycin, daunorubicin, doxorubicin, bleomycin sulfate, plicamycin, mitomycin; enzymes, including L-asparaginase; platinum coordination complexes, including cisplatin, carboplatin; hydroxyurea, procarbazine, mitotane; and hormones or related agents, including adrenocorticosteroids such as prednisone and prednisolone; aminoglutethimide; progestins such as hydroxyprogesterone caproate, medroxyprogesterone acetate, megestrol acetate, estrogens and androgens such as diethylstilbestrol, fluoxymesterone, ethynyl estradiol, antiestrogens such as tamoxifen, and gonadotropin-releasing hormone analogs such as leuprolide.

The present invention also provides kits for use in treating cancer comprising an administration means and a container means containing one or more cytokine-expressing polynucleotide constructs in a sterile environment. Also provided are kits for use in treating cancer comprising an administration means and a container means containing one or more cytokine-expressing polynucleotide constructs and one or more cationic compounds in a sterile environment. Examples of cationic compounds are described above. The cytokine-expressing polynucleotide constructs and the cationic compounds may be in the same container means or in separate container means. Preferably, the polynucleotide construct is in the amount of 1 ng to 20 mg.

Container means include glass containers, plastic containers, or strips of plastic or paper. In one embodiment, the container means is a syringe and the administration means is a plunger. In another embodiment, the administration means is a catheter.

The cytokine encoded by the polynucleotide construct of the kit of the present invention can be an IFN ω and one or more additional cytokines, including any of the cytokines described herein. Preferably, the cytokine is IFN ω and/or an IFN α . The construct can be in the form of a pharmaceutical composition and can contain a pharmaceutically acceptable carrier.

Pharmaceutical compositions are described above. The kit can further comprise a pharmaceutically acceptable carrier in a separate container means.

The kit can further comprise an instruction sheet for administration of the composition into a mammal. The components of the polynucleotide composition are preferably provided as a liquid solution, such as a suspension, a solution, or an emulsion; or in lyophilized form as a dried powder or a cake. If the polynucleotide construct is provided in lyophilized form, preferably the kit further comprises a container means containing a suitable vehicle, such as sterile pyrogen-free water, for reconstitution of the lyophilized polynucleotide construct, or any buffer described herein, including PBS, normal saline, Tris buffer, and sodium phosphate vehicle.

The term "cytokine" refers to polypeptides, including but not limited to interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, and IL-18), α interferons (*e.g.*, IFN α), β interferons (*e.g.*, IFN β), γ interferons (*e.g.*, IFN γ), ω interferon (IFN ω), τ interferons (IFN τ), colony stimulating factors (CSFs, *e.g.*, CSF-1, CSF-2, and CSF-3), granulocyte-macrophage colony stimulating factor (GM-CSF), epidermal growth factor (EGF), fibroblast growth factors (FGFs, *e.g.*, acidic fibroblast growth factor, basic fibroblast growth factor, FGF-1, FGF-2, FGF-3, FGF-4, and FGF-5), transforming growth factor (TGF, *e.g.*, TGF α and TGF β), platelet-derived growth factor (PDGF), tumor necrosis factors (TNFs, *e.g.*, TNF- α and TNF- β), and insulin-like growth factors (IGFs, *e.g.*, IGF-I and IGF-II).

A "polypeptide" refers to any translation product of a polynucleotide, regardless of the size of the translation product, and regardless of whether the translation product is post-translationally modified (*e.g.*, glycosylated) or not.

The polynucleotide construct of the present invention, whether complexed with cationic vehicle or not, can be administered by any suitable route of administration, including intramuscularly, subcutaneously, intravenously, transdermally, intranasally, by inhalation, or transmucosally (*i.e.*, across a mucous membrane). Similarly, the pharmaceutical composition of the present invention can be administered by any suitable route, including

intramuscularly, into or near a tumor, into a cavity (*e.g.*, intraperitoneally), subcutaneously, intravenously, transdermally, intranasally, by inhalation, or transmucosally (*i.e.*, across a mucous membrane).

Any mode of administration can be used so long as the mode results in the expression of one or more cytokines in an amount sufficient to decrease the tumorigenicity of the cancer bearing mammal. This includes needle injection, catheter infusion, biolistic injectors, particle accelerators (*i.e.*, "gene guns"), pneumatic "needleless" injectors (*e.g.*, MedEJet, PedeJet, Bioject), gelfoam sponge depots, other commercially available depot materials, osmotic pumps (*e.g.*, Alza minipumps), oral or suppositorial solid (tablet or pill) pharmaceutical formulations, and decanting or topical applications during surgery. Preferred methods include needle injection and catheter infusion.

A "polynucleotide construct" is a polynucleotide molecule that carries genetic information for encoding one or more molecules, preferably, cytokines. The polynucleotide material delivered to the cells *in vivo* can take any number of forms. It can contain the entire sequence or only a functionally active fragment of a cytokine gene.

The polynucleotide construct comprises at least one polynucleotide (*e.g.*, DNA, RNA, ribozyme, phosphorothioate, or other modified nucleic acid) encoding one or more molecules. Preferred molecules are cytokines. The polynucleotide can be provided in linear, circular (*e.g.* plasmid), or branched form; and double-stranded or single-stranded form. The polynucleotide can involve a conventional phosphodiester bond or a non-conventional bond (*e.g.*, an amide bond as in peptide nucleic acid (PNA)). The choice of polynucleotide encoding a cytokine will depend on the desired kinetics and duration of expression. When long term delivery of the polynucleotide construct is desired, the preferred polynucleotide is DNA. Alternatively, when short term delivery is desired, the preferred polynucleotide is mRNA. RNA will be rapidly translated into polypeptide, but will be degraded by the target cell more quickly than DNA. In general, because of the greater resistance of circular DNA molecules to nucleases, circular DNA molecules will persist longer than linear polynucleotides, and

they will be less likely to cause insertional mutation by integrating into the target genome.

In one embodiment, the polynucleotide sequence encoding one or more cytokines is RNA. Most preferably, the RNA is messenger RNA (mRNA). Methods for introducing RNA sequences into mammalian cells is described in U.S. patent No. 5,580,859. A viral alphavector, a non-infectious vector useful for administering RNA, may be used to introduce RNA into mammalian cells. Methods for the *in vivo* introduction of alphaviral vectors to mammalian tissues are described in Altman-Hamamdzc, S., *et al.*, *Gene Therapy* 4: 815-822 (1997). Preferably, the polynucleotide sequence encoding one or more cytokines is DNA. In a DNA construct, a promoter is preferably operably linked to the polynucleotide encoding a cytokine. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, can be included in the polynucleotide construct to direct cell-specific transcription of the DNA.

An operable linkage is a linkage in which a polynucleotide sequence encoding a cytokine is connected to one or more regulatory sequence in such a way as to place expression of the cytokine sequence under the influence or control of the regulatory sequence(s). Two DNA sequences (such as a coding sequence and a promoter region sequence linked to the 5' end of the coding sequence) are operably linked if induction of promoter function results in the transcription of mRNA encoding the desired polypeptide and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the polypeptide, antisense RNA, or (3) interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably linked to a DNA sequence if the promoter was capable of affecting transcription of that DNA sequence.

Preferably, the polynucleotide construct is a circular or linearized plasmid containing non-infectious nucleotide sequence. A linearized plasmid

is a plasmid that was previously circular but has been linearized, for example, by digestion with a restriction endonuclease. The polynucleotide sequence encoding a cytokine may comprise a sequence which directs the secretion of the polypeptide.

5 "Non-infectious" means that the polynucleotide construct does not infect mammalian cells. Thus, the polynucleotide construct can contain functional sequences from non-mammalian (*e.g.*, viral or bacterial) species, but the construct does not contain non-mammalian nucleotide sequences that facilitate infection of the construct into mammalian cells.

10 "Non-integrating" means that the polynucleotide construct does not integrate into the genome of mammalian cells. The construct can be a non-replicating DNA sequence, or specific replicating sequences genetically engineered to lack the ability to integrate into the genome. The polynucleotide construct does not contain functional sequences that facilitate integration of the cytokine-encoding polynucleotide sequence into the genome of mammalian cells.

15 The polynucleotide construct is assembled out of components where different selectable genes, origins, promoters, introns, 5' untranslated (UT) sequence, terminators, polyadenylation signals, 3' UT sequence, and leader peptides, etc. are put together to make the desired vector. The precise nature of the regulatory regions needed for gene expression can vary between species or cell types, but shall in general include, as necessary, 5' non-transcribing and 5' non-translating (non-coding) sequences involved with initiation of transcription and translation respectively, such as the TATA box, capping sequence, 20 CAAT sequence, and the like, with those elements necessary for the promoter sequence being provided by the promoters of the invention. Such transcriptional control sequences can also include enhancer sequences or upstream activator sequences, as desired.

25 The polynucleotide construct can be an expression vector. A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the polypeptide coding sequence, and signals required for the termination of transcription and polyadenylation of the 30

transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRS) from retroviruses, *e.g.*, RSV, HTLVI, HIVI, MPSV and the immediate early promoter of the cytomegalovirus (CMV IEP). However, cellular elements can also be used (*e.g.*, the human actin promoter, metallothionein promoter). In humans, CMV IEP is preferred. Suitable expression vectors for use in practicing the present invention include, for example, vectors such as PSVL and PMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109), VR1012, VR1055, and pcDNA3 (Invitrogen, San Diego, CA). All forms of DNA, whether replicating or non-replicating, which do not become integrated into the genome, and which are expressible, are within the methods contemplated by the invention.

The vector containing the DNA sequence (or the corresponding RNA sequence) which can be used in accordance with the invention can be a eukaryotic expression vector. Techniques for obtaining expression of exogenous DNA or RNA sequences in a host are known. See, for example, Korman, *et al.*, *Proc. Nat. Acad. Sci. (USA)* 84:2150-2154 (1987).

Secretion of a cytokine from a cell can be facilitated by a leader or secretory signal sequence. In a preferred embodiment, either the native leader sequence of a cytokine is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the peptide that is operably linked to it. Alternatively, a heterologous mammalian leader sequence, or a functional derivative thereof, may be used. For example, the wild-type leader sequence may be substituted with the leader sequence of human tissue plasminogen activator or mouse β -glucuronidase.

For the methods of the present invention, a single polynucleotide construct containing more than one polynucleotide sequences encoding one or more molecules, or more than one polynucleotide constructs each containing polynucleotide sequences encoding one or more molecules may be co-injected or sequentially injected. For example, a single polynucleotide construct

containing one polynucleotide encoding an interferon and another polynucleotide encoding an additional cytokine or an immunomodulatory molecule, *i.e.*, MHC class I antigen, tumor antigen, and co-stimulatory molecule, can be injected. Alternatively, two polynucleotide construct can be injected where one encodes a cytokine to enhance anti-tumor efficacy of the other gene product. For example, an IFN ω , IFN α , IL-12 or IL-2-expressing polynucleotide construct can be co-injected with a polynucleotide construct encoding a different cytokine. More specifically, an IL-2 expressing plasmid could be co-injected with a G-CSF or GM-CSF expressing plasmid. Alternatively, one or more plasmids could be administered initially and other plasmid(s) could be administered subsequently at various time intervals. Combination of the present invention with therapeutic agents such as lymphokine-activated killer cells (LAK) and tumor-infiltrating lymphocytes (TIL) is also envisioned.

It will be recognized in the art that some amino acid sequences of the polypeptides described herein can be varied without significant effect on the functional activity of the polypeptides. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the polypeptide which determine activity. Such variations include deletions, insertions, inversions, repeats, and type substitutions. Guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," (*Science* 247:1306-1310 (1990)). Compositions within the scope of the invention can be assayed according to the antiproliferation assay described herein. Amino acids that are critical for cytokine activity can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith, *et al.*, *J. Mol. Biol.* 224:899-904 (1992) and de Vos, *et al.* *Science* 255:306-312 (1992)).

The present invention further relates to using variants of the cytokine-encoding polynucleotide, which encode portions, analogs or derivatives of the cytokine. Variants may occur naturally, such as a natural allelic variant. By an

"allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

5 Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the cytokine or portions thereof. Also especially preferred in this regard are conservative substitutions. For example, aromatic amino acids that can be conservatively substituted for one another include phenylalanine, tryptophan, and tyrosine. Hydrophobic amino acids that can be conservatively substituted for one another include leucine, isoleucine, and valine. Polar amino acids that can be conservatively substituted for one another include glutamine and asparagine. Basic amino acids that can be conservatively substituted for one another include arginine, lysine, and histidine. Acidic amino acids that can be conservatively substituted for one another include aspartic acid and glutamic acid. Small amino acids that can be conservatively substituted for one another include alanine, serine, threonine, methionine, and glycine.

20 Substitutions, deletions, or insertions can be made outside of the region encoding the shortest active fragment of the cytokine, without affecting the activity of the cytokine. Further, mutated proteins (or muteins) often retain a biological activity that is similar to that of the naturally occurring protein. For example, Gayle and coworkers (*J. Biol. Chem.* 268: 22105-22111 (1993)) conducted an extensive mutational analysis of the human cytokine IL-1 α . They used random mutagenesis to generate over 3,500 individual IL-1 α mutants with an average of 2.5 amino acid changes per mutein over the entire length of the molecule. Multiple mutations were examined at every possible amino acid and, on average, each mutein's amino acid sequence was 98.4% identical to that of naturally occurring IL-1 α . The investigators observed that most of the molecule could be mutated with little effect on either binding or

biological activity, and that 75% of the molecule may not contribute significantly to the biological activity of the molecule.

Similarly, Gronenborn and colleagues (*FEBS Letters* 231: 135-138 (1988)) analyzed the receptor binding activity of six mutant IL-1 α polypeptides. Each mutant contained a single amino acid alteration from the naturally occurring IL-1 α polypeptide and was examined under four sets of experimental conditions. In this study, the investigators found very little difference between the receptor binding activity of the mutants and naturally occurring IL-1 α .

Further, Zurawski and colleagues (*EMBO J.* 12: 5113-5119 (1993)) studied residues 41-142 of mIL-2 by generating 1,090 muteins. The extent of the mutagenesis was such that there was an average of 11 different amino acid substitutions per naturally occurring amino acid residue, with the exception of the extreme N- and C-termini and residues 31-40. The mIL-2 muteins were assayed for specific activity and compared to that of naturally occurring mIL-2. The degree to which the specific activity was antagonized by a previously characterized mIL-2 mutant was also assessed. The investigators observed that in the 149 residue mIL-2 protein, only 23 residues are important for interaction with IL-2R, 18 residues are presumed to be part of the structural core, and three additional residues are important for structure. 98 mIL-2 residues (or 65% of the protein) were assigned as relatively unimportant residues.

The polynucleotide and amino acid sequences encoding an IFN ω include the sequences for the complete IFN ω and the mature IFN ω set forth in U.S. Patent No. 4,917,887; European Patent Publication No. 0 170 204 B1; and Capon, D.J., *et al.*, *Molec. Cell. Biol.* 5: 768-779 (1985); Hauptmann, R. and P. Swetly, *Nucl. Acids Res.* 13: 4739-4749 (1985); Adolf, G.R., *et al.*, *Biochim. Biophys. Acta* 1089: 167-174 (1991); Mege, D., *et al.*, *J. Interf. Res.* 11: 341-350 (1991); Charlier, M., *et al.*, *J. Interf. Res.* 13: 313-322 (1993); Hughes, A.L., *J. Mol. Evol.* 41: 539-548 (1995); and Roberts, R.M., *et al.*, *Prog. Nucl. Acid Res. Molec. Biol.* 56:287-325, edited by W.E. Cohn, Academic Press (1997).

The polynucleotide and amino acid sequences encoding IFN α include the sequences for the complete IFN α and the mature IFN α set forth in U.S. Patent Nos. 4,530,901; 4,695,543; 4,695,623; 4,748,233; 4,892,743; 4,897,471; 4,973,479; 4,975,276; and 5,098,703; and in Pestka, S., *Methods Enzymol.* 119: 3-14 (1986); Hughes, A.L., *J. Mol. Evol.* 41: 539-548 (1995); and Roberts, R.M., *et al.*, *Prog. Nucl. Acid Res. Molec. Biol.* 56:287-325, edited by W.E. Cohn, Academic Press (1997).

The polynucleotide and amino acid sequences encoding an IL-2 include the sequences for the complete human IL-2 and mature IL-2 set forth in Lupker, J. *et al.*, EP 0307285-A3 (1989), U.S. Patent No. 5,641,665, Maeda *et al.*, *Biochem. Biophys. Res. Commun.* 115:1040-1047 (1983), Mita *et al.*, *Biochem. Biophys. Res. Commun.* 117:114-121 (1983), Taniguchi *et al.*, *Nature* 302:305-310 (1983), Devos *et al.*, *Nucleic Acid Res.* 11:4307-4323 (1983), Fujita *et al.*, *Proc. Natl. Acad. Sci. USA* 80:74347-7441 (1983), Clark *et al.*, *Proc. Natl. Acad. Sci. USA* 81:2543-2547 (1984) and Cullen, *DNA* 7:645-650 (1988).

The polynucleotide sequences encoding an IFN ω , an IFN α , and an IL-2 also include sequences that encode the complete polypeptide encoded by the nucleotide sequences set forth in SEQ ID Nos. 7, 9 and 13, respectively, and the mature polypeptides encoded by nucleotide sequences set forth in SEQ ID Nos. 7, 9 and 13, respectively. The polynucleotide sequences encoding IL-2 further includes the sequence that encodes the complete IL-2 polypeptide encoded by the nucleotide sequence set forth in SEQ ID No. 25, shown as SEQ ID No. 26.

Thus, a polynucleotide sequence encoding a polypeptide of the present invention can encode a polypeptide having one to twenty amino acid substitutions, deletions or insertions, either from natural mutations or human manipulation, relative to the full length or mature IFN α , IFN ω , or IL-2. Preferably, no more than one to fifteen substitutions, deletions or insertions are present, relative to the full length or mature IFN α , IFN ω , or IL-2 (excluding the signal sequence). More preferably, no more than one to ten

substitutions, deletions or insertions are present. Still more preferably, no more than one to five substitutions, deletions or insertions are present.

Determining an effective amount of substance to be delivered can depend upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the mammal, the precise condition requiring treatment and its severity, and the route of administration. The precise amount, number of doses, and timing of doses will be determined by the attending physician or veterinarian.

If the polynucleotide construct of the present invention is administered as a pharmaceutical composition, the pharmaceutical composition can be formulated according to known methods for preparing pharmaceutical compositions, whereby the substance to be delivered is combined with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their preparation are described, for example, in Remington's Pharmaceutical Sciences, 16th Edition, A. Osol, Ed., Mack Publishing Co., Easton, PA (1980), and Remington's Pharmaceutical Sciences, 19th Edition, A.R. Gennaro, Ed., Mack Publishing Co., Easton, PA (1995).

The pharmaceutical composition can be in the form of an emulsion, gel, solution, suspension, or other form known in the art. Optionally, it can contain one or more lipids as described above. In addition, the pharmaceutical composition can also contain pharmaceutically acceptable additives including, for example, diluents, binders, stabilizers, and preservatives. Administration of pharmaceutically acceptable salts of the polynucleotides described herein is preferred. Such salts can be prepared from pharmaceutically acceptable non-toxic bases including organic bases and inorganic bases. Salts derived from inorganic bases include sodium, potassium, lithium, ammonium, calcium, magnesium, and the like. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, basic amino acids, and the like.

For aqueous pharmaceutical compositions used *in vivo*, sterile pyrogen-free water is preferred. Such formulations will contain an effective amount of the substance together with a suitable amount of vehicle in order to

prepare pharmaceutically acceptable compositions suitable for administration to a human or animal.

5 A pharmaceutical composition can be in solution form, or alternatively, in lyophilized form for reconstitution with a suitable vehicle, such as sterile, pyrogen-free water. Both liquid and lyophilized forms will comprise one or more agents, preferably buffers, in amounts necessary to suitably adjust the pH of the injected solution.

10 The container in which the pharmaceutical formulation is packaged prior to use can comprise a hermetically sealed container enclosing an amount of the lyophilized formulation or a solution containing the formulation suitable for a pharmaceutically effective dose thereof, or multiples of an effective dose. The pharmaceutical formulation is packaged in a sterile container, and the hermetically sealed container is designed to preserve sterility of the pharmaceutical formulation until use. Optionally, the container can be
15 associated with administration means and or instruction for use.

It will be apparent to one skilled in the art, in view of the following detailed description and the claims appended hereto, that various substitutions and modifications may be made to the present invention without departing from the scope of the invention as claimed.

20 The present invention is broadly directed to compositions and methods for improving the effectiveness of polypeptide delivery into a vertebrate by administering a polynucleotide encoding the polypeptide to the vertebrate's cells *in vivo*. Such compositions comprise a polypeptide-encoding polynucleotide and sodium phosphate dissolved in an aqueous solution. In
25 one preferred embodiment, about 1 ng to about 30 mg of a polynucleotide is dissolved in a solution of about 20 mM to about 300 mM sodium phosphate. In another preferred embodiment, about 1 ng to about 30 mg of a polynucleotide is associated with a cationic lipid in an aqueous solution of about 0.1 to about 150 mM sodium phosphate. In this embodiment, the
30 aqueous solution is substantially free of chloride anion.

In this manner, the present invention provides a method of enhancing the level of polypeptide expression from delivered polynucleotides *in vivo*

and/or facilitating uptake of the polynucleotides by vertebrate cells. Delivery methods utilizing the compositions of the present invention significantly enhance the levels of *in vivo* transfection and *in vivo* polypeptide expression compared with traditional methods, *i.e.*, delivery of a polypeptide-encoding polynucleotide in a solution of normal saline (about 154 mM NaCl) or phosphate buffered saline ("PBS": about 154 mM NaCl, about 10 mM sodium phosphate at pH 7).

It is to be noted that the term "a" or "an" entity, refers to one or more of that entity; for example, "a polynucleotide," is understood to represent one or more polynucleotides. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

The term "vertebrate" is intended to encompass a singular "vertebrate" as well as plural "vertebrates," and comprises mammals and birds, as well as fish, reptiles, and amphibians.

The terms "saline" or "normal saline" as used herein refer to an aqueous solution of about 145 mM to about 155 mM sodium chloride, preferably about 154 mM sodium chloride. The terms "phosphate buffered saline" or "PBS" refer to an aqueous solution of about 145 mM to about 155 mM sodium chloride, preferably about 154 mM sodium chloride, and about 10 mM sodium phosphate, at a pH ranging from about 6.0 to 8.0, preferably at a pH ranging from about 6.5 to about 7.5, most preferably at pH 7.0.

As used herein, an "ion," an "ionic molecule," or an "ionic compound" refers to a charged molecule or compound. A "cation," a "cationic molecule," or a "cationic compound" refers to a positively charged molecule or compound, for example Na^+ . An "anion," an "anionic molecule," or an "anionic compound" refers to a negatively charged molecule or compound, for example, HPO_4^{2-} or H_2PO_4^- . Cations and anions may have any number of positive or negative charges, respectively, *i.e.*, they may be monovalent like Na^+ or multivalent, *e.g.*, divalent such as Ca^{2+} , or trivalent such as Al^{3+} . Anions can be either inorganic or organic, examples of the latter being pyruvate or citrate anions.

Salts and Associated Formulations

Certain embodiments of the present invention comprise the salt sodium phosphate. As used herein, a "salt" of the present invention is a compound having a positively charged cation, C, and a negatively charged anion, A. A salt may be in a solid crystalline form, but preferably for the present invention, is dissolved in an aqueous solution, *i.e.*, liquid water. As used herein, "sodium phosphate" can be either the monobasic form, *e.g.*, Na_2HPO_4 , or the dibasic form, *e.g.*, NaH_2PO_4 , but a mixture of the two, resulting in a desired pH, is most preferred. Accordingly, as used herein the term "sodium phosphate" refers to a mixture of the dibasic and monobasic forms of the salt to reach a given pH.

The pH values for the sodium phosphate solution of the present invention can range from about pH 4 to about pH 10, depending on the properties of the particular salt solution. These pH values include about pH 4, about pH 4.5, about pH 5, about pH 5.5, about pH 6, about pH 6.5, about pH 7, about pH 7.5, about pH 8, about pH 8.5, about pH 9, about pH 9.5, and about pH 10. As used herein, the term "about" when referring to pH values indicates that the pH value may vary by as much as 0.4 pH units in either direction due to, for example, standard error or equipment error. Preferred pH values for a solution of sodium phosphate are from about pH 6 to about pH 8. More preferred pH values for a solution of sodium phosphate range from about pH 6.5 to about pH 7.5. Even more preferred pH values for a solution of sodium phosphate or potassium phosphate range from about 6.8 to about 7.4.

Sodium phosphate is preferably dissolved in aqueous solution at a concentration which enhances entry of a polypeptide-encoding polynucleotide into vertebrate cells *in vivo*, and/or enhances polypeptide expression, relative to saline, PBS, or water.

Certain embodiments of the present invention are drawn to compositions comprising about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide

within vertebrate cells *in vivo*; and sodium phosphate dissolved in the aqueous solution at a molar concentration from about 20 mM to about 300 mM, and reaction, association, or dissociation products thereof. The present invention is further drawn to methods to use such a composition, methods to make such a composition, and pharmaceutical kits.

The term "sodium phosphate dissolved in aqueous solution at a given molar concentration" means that the molecular mass, *e.g.*, in grams, of a crystallized form of sodium phosphate required to produce a given volume of a solution of a given molar concentration is calculated based on the molecular weight of the particular crystalline form. That amount of crystals is weighed out using standard laboratory procedures, the crystals are added to a volume of water or other aqueous solution which is slightly less than the final desired volume, the liquid is mixed until the crystals are fully dissolved, and then the volume of the liquid is brought up to the final desired volume.

The term "reaction, association, or dissociation products thereof" refers to any ionic interactions which may be formed in the aqueous solution once the salt crystals are dissolved. For example, once sodium phosphate is dissolved in an aqueous solution, the cations and anions disassociate and are free in solution to interact with other cations or anions that may be present in the solution, including, for example, a negatively-charged polynucleotide molecule. The interactions taking place in a complex aqueous sodium phosphate solution, except for the precipitation of insoluble salt complexes, are transient and reversible, and cannot be precisely predicted at any point in time. Therefore, once an aqueous solution comprising sodium phosphate at a certain molar concentration is prepared, the interactions in the solution may include reaction products other than the interaction of the cation and anion which composed the sodium phosphate crystals that were added to the solution as described above.

Preferably in the present embodiment, sodium phosphate is dissolved in aqueous solution at a molar concentration ranging from about 25 mM to about 290 mM, from about 30 mM to about 280 mM, from about 35 mM to about 270 mM, from about 40 mM to about 260 mM, from about 45 mM to

about 255 mM, from about 50 mM to about 250 mM, from about 55 mM to about 245 mM, from about 60 mM to about 240 mM, from about 65 mM to about 235 mM, from about 70 mM to about 230 mM, from about 75 mM to about 225 mM, from about 80 mM to about 220 mM, from about 85 mM to about 215 mM, from about 90 mM to about 210 mM, from about 95 mM to about 205 mM, from about 100 mM to about 200 mM, from about 105 mM to about 195 mM, from about 110 mM to about 190 mM, from about 115 mM to about 185 mM, from about 120 mM to about 180 mM, from about 125 mM to about 175 mM, from about 130 mM to about 170 mM, from about 135 mM to about 165 mM, from about 140 mM to about 160 mM, and from about 145 mM to about 155 mM.

More preferably in the present embodiment, sodium phosphate is dissolved in aqueous solution at a molar concentration of about 20mM, about 25 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM, about 50 mM, about 55 mM, about 60 mM, about 65 mM, about 70 mM, about 75 mM, about 80 mM, about 85 mM, about 90 mM, about 95 mM, about 100 mM, about 105 mM, about 110 mM, about 115 mM, about 120 mM, about 125 mM, about 130 mM, about 135 mM, about 140 mM, about 145 mM, about 150 mM, about 155 mM, about 160 mM, about 165 mM, about 170 mM, about 175 mM, about 180 mM, about 185 mM, about 190 mM, about 200 mM, about 205 mM, about 210 mM, about 215 mM, about 220 mM, about 225 mM, about 230 mM, about 235 mM, about 240 mM, about 245 mM, about 250 mM, about 255 mM, about 260 mM, about 265 mM, about 270 mM, about 275 mM, about 280 mM, about 285 mM, about 290 mM, about 295 mM, and about 300 mM.

Even more preferably in the present embodiment, sodium phosphate is dissolved in aqueous solution at a molar concentration of about 100 to about 200 mM. Most preferably, sodium phosphate is dissolved in aqueous solution at a molar concentration of about 150 mM.

As used herein, a phrase such as "a molar concentration of *about* 150 mM" refers to the range of molar concentrations *approaching* 150 mM to the best approximation obtainable by one of ordinary skill in the art using standard

laboratory equipment and methods. For example, "the salt sodium phosphate dissolved in aqueous solution of about 150 mM," prepared using ordinary laboratory balances and measuring glassware, and using generally accepted techniques, may range anywhere from approximately 145 mM to approximately 155 mM based on the standard error inherent in preparing chemical solutions. Such a standard error range would be well understood by one of ordinary skill in the art to be equivalent to "about 150 mM."

According to the present embodiment, compositions comprising about 1 ng to about 30 mg of a polynucleotide in aqueous solution and sodium phosphate dissolved in the aqueous solution at a molar concentration from about 20 mM to about 300 mM as described above may further comprise chloride ion, represented by the symbol Cl^- , in the aqueous solution at a molar equivalent concentration of 0 (zero) mM to about 125 mM. By "molar equivalent" is meant the molar concentration of chloride ion in solution, as opposed to the molar concentration of the salt added to the aqueous solution. For example, chloride ion may be added to the aqueous solution as part of certain salt crystals dissolved in the solution, such as sodium chloride (NaCl) or calcium chloride (CaCl_2). Each mole of sodium chloride crystals added to an aqueous solution will add one mole equivalent of chloride ion to the solution, where each mole of calcium chloride crystals added to an aqueous solution will add two mole equivalents of chloride ion to the solution. Alternatively, chloride ion may be added to the aqueous solution as part of an acid or base such as hydrogen chloride or ammonium chloride.

Preferably, chloride ion is present in the aqueous solution at a molar equivalent concentration ranging from 0 mM to about 120 mM, from 0 mM to about 115 mM, from 0 mM to about 110 mM, from 0 mM to about 105 mM, from 0 mM to about 100 mM, from 0 mM to about 95 mM, from 0 mM to about 90 mM, from 0 mM to about 85 mM, from 0 mM to about 80 mM, from 0 mM to about 75 mM, from 0 mM to about 70 mM, from 0 mM to about 65 mM, from 0 mM to about 60 mM, from 0 mM to about 55 mM, from 0 mM to about 50 mM, from 0 mM to about 45 mM, from 0 mM to about 40 mM, from 0 mM to about 35 mM, from 0 mM to about 30 mM, from 0 mM to about 25

mM, from 0 mM to about 20 mM, from 0 mM to about 15 mM, from 0 mM to about 10 mM, or from 0 mM to about 5 mM.

More preferably, chloride ion is present in the aqueous solution at a molar concentration of about 120 mM, about 115 mM, about 110 mM, about 105 mM, about 100 mM, about 95 mM, about 90 mM, about 85 mM, about 80 mM, about 75 mM, about 70 mM, about 65 mM, about 60 mM, about 55 mM, about 50 mM, about 45 mM, about 40 mM, about 35 mM, about 30 mM, about 25 mM, about 20 mM, about 15 mM, about 10 mM, about 5 mM, or 0 mM.

Most preferably, the aqueous solution is substantially free of chloride ion. As used herein, the phrase "substantially free of chloride ion" indicates that the amount of chloride ion added into the composition is insubstantial and that the addition cannot alter the transcription- and/or expression-enhancing activity of the composition at a significant level. The phrase "essentially free of chloride ion" indicates that no chloride ion is intentionally added to the composition. However, some chloride ion may be present due to, for example, impurities in other components of the composition.

In certain embodiments, described in more detail below, compositions of the present invention may further comprise one or more transfection facilitating materials including, but not limited to, materials such as cationic lipids, calcium phosphate, alum, gold, tungsten, or other metal particles, peptides, proteins, and polymers. However, compositions of the present embodiment, which comprise a polynucleotide in aqueous solution and sodium phosphate dissolved in the aqueous solution at a molar concentration from about 20 mM to about 300 mM as described above, are preferably free of cationic lipids.

Accordingly a preferred embodiment of the present invention is a composition comprising: about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; sodium phosphate dissolved in the aqueous solution at a molar concentration from about 20 mM to about 300 mM, and

reaction, association, or dissociation products thereof; where the composition is free of cationic lipids.

Further embodiments of the present invention are drawn to compositions comprising: about 1 ng to about 30 mg of a polynucleotide in aqueous solution, where the polynucleotide operably encodes a polypeptide within vertebrate cells *in vivo*; sodium phosphate dissolved in the aqueous solution at a molar concentration from about 0.1 mM to about 150 mM, and reaction, association, or dissociation products thereof; a cationic lipid suspended in said aqueous solution; and where the aqueous solution is substantially free of chloride anion. The present invention is further drawn to methods to use such a composition, methods to make such a composition, and pharmaceutical kits.

Preferably, sodium phosphate is dissolved in aqueous solution at a molar concentration ranging from about 0.1 mM to about 145 mM, from about 0.1 mM to about 140 mM, from about 0.1 mM to about 135 mM, from about 0.1 mM to about 130 mM, from about 0.1 mM to about 125 mM, from about 1 mM to about 120 mM, from about 1 mM to about 115 mM, from about 1 mM to about 110 mM, from about 1 mM to about 105 mM, from about 1 mM to about 100 mM, from about 1 mM to about 95 mM, from about 1 mM to about 90 mM, from about 1 mM to about 85 mM, from about 1 mM to about 80 mM, from about 1 mM to about 75 mM, from about 1 mM to about 70 mM, from about 1 mM to about 65 mM, from about 1 mM to about 60 mM, from about 1 mM to about 55 mM, from about 1 mM to about 50 mM, from about 1 mM to about 45 mM, from about 1 mM to about 40 mM, from about 1 mM to about 35 mM, from about 1 mM to about 30 mM, from about 1 mM to about 25 mM, from about 1 mM to about 20 mM, from about 1 mM to about 15 mM, from about 1 mM to about 10 mM, from about 1 mM to about 5 mM, from about 1 mM to about 4.0 mM, from about 1 mM to about 3.0 mM, or from about 1 mM to about 2.5 mM.

More preferably, sodium phosphate is dissolved in aqueous solution at a molar concentration of about 145 mM, about 140 mM, about 135 mM, about 130 mM, about 125 mM, about 120 mM, about 115 mM, about 110 mM,

about 105 mM, about 100 mM, about 95 mM, about 90 mM, about 85 mM,
about 80 mM, about 75 mM, about 70 mM, about 65 mM, about 60 mM,
about 55 mM, about 50 mM, about 45 mM, about 40 mM, about 35 mM,
about 30 mM, about 25 mM, about 20 mM, about 15 mM, about 10 mM,
5 about 5 mM, about 4.0 mM, about 3.0 mM, about 2.5 mM, about 2.0 mM,
about 1.5 mM, about 1.0 mM, about 0.5 mM, or about 0.1 mM.

Even more preferably, sodium phosphate is dissolved in aqueous
solution at a molar concentration of about 1 mM to 10 mM, with about 2.5
mM being most preferred.

10 Those embodiments of the present invention comprising sodium
phosphate dissolved in the aqueous solution at a molar concentration from
about 0.1 mM to about 150 mM, and which are substantially free of chloride
ion also comprise a cationic lipid suspended in the aqueous solution. Cationic
lipids are described in more detail above. While not being bound by theory,
15 cationic lipids are thought to interact with anionic polynucleotide molecules in
solution, and the complexes formed thereby are thought to have an improved
ability to enter into vertebrate cells.

That sodium phosphate and sodium phosphate solutions disclosed
herein were effective in increasing expression of polypeptides encoded by
polynucleotides delivered to vertebrate cells *in vivo* relative to saline or PBS
was unexpected in view of results showing that other salt solutions have no
enhancing effect on expression of polypeptides encoded by polynucleotides
delivered to vertebrate cells *in vivo*, or even hinder such expression. For
20 example, the following salt solutions had no ability to enhance, relative to a
salt solution consisting essentially of normal saline, the entry of
polynucleotides into vertebrate cells *in vivo*, and/or the *in vivo* expression of
polypeptides encoded by such polynucleotides: 150 mM potassium chloride,
150 mM magnesium chloride, 150 mM calcium chloride, 150 mM zinc
chloride, 150 mM ferrous chloride, 150 mM magnesium phosphate, 150 mM
25 calcium phosphate, 150 mM aluminum phosphate, 150 mM ferric phosphate,
30 150 mM sodium citrate, and 150 mM sodium oxalate. See Table 10.

Polynucleotides

The present invention covers the delivery to a vertebrate of a polypeptide-encoding polynucleotide in a detectable amount. Preferably, the encoded polypeptide is expressed *in vivo* in the vertebrate in an amount
5 sufficient to provide an immunogenic, immunomodulatory, therapeutic, or corrective effect to a vertebrate in need of such treatment.

The term "nucleic acid" is intended to encompass a singular "nucleic acid" as well as plural "nucleic acids," and refers to an isolated molecule or construct, *e.g.*, virus genomes (preferably non-infectious), messenger RNA
10 (mRNA), plasmid DNA (pDNA), or derivatives of pDNA (*e.g.*, minicircles as described in (Darquet, A-M *et al.*, *Gene Therapy* 4:1341-1349 (1997)) comprising a polynucleotide. A nucleic acid may be provided in linear (*e.g.*, mRNA), circular (*e.g.*, plasmid), or branched form as well as double-stranded
15 or single-stranded forms. A nucleic acid may comprise a conventional phosphodiester bond or a non-conventional bond (*e.g.*, an amide bond, such as found in peptide nucleic acids (PNA)).

The term "polynucleotide" refers to any one or more nucleic acid segments, *e.g.*, DNA or RNA fragments, present in a nucleic acid or construct.
20 Two or more polynucleotides of the present invention can be present in a single nucleic acid construct, *e.g.*, on a single plasmid, or in separate nucleic acid constructs, *e.g.*, on separate plasmids. Furthermore, any polynucleotide may encode a single polypeptide, *e.g.*, a single antigen, cytokine, or regulatory polypeptide, or may encode more than one polypeptide, *e.g.*, a polynucleotide
25 may encode two or more polypeptides. In addition, a polynucleotide may encode a regulatory element such as a promoter or a transcription terminator, or may encode a specific element of a polypeptide or protein, such as a secretory signal peptide or a functional domain.

Nucleic acids and/or polynucleotides of the present invention, *e.g.*,
30 plasmid DNA, derivatives of plasmid DNA, mRNA, linear DNA, viral genomes, or polynucleotide fragments contained therein may be formulated into any of the various compositions and may be used in any of the methods

disclosed herein. For aqueous compositions used *in vivo*, use of sterile pyrogen-free water is preferred. Such formulations will contain an effective amount of a polynucleotide together with sodium phosphate as disclosed herein, in order to prepare pharmaceutically acceptable compositions suitable for optimal administration to a vertebrate. Insoluble polynucleotides may be solubilized in a weak acid or weak base, and then diluted to the desired volume, for example, with an aqueous solution of the present invention. The pH of the solution may be adjusted as appropriate. In addition, a pharmaceutically acceptable additive can be used to provide an appropriate osmolality. Such additives are within the purview of one skilled in the art.

The amount of a polynucleotide included in a composition of the present invention depends on many factors, including the age and weight of the subject, the delivery method and route, the type of treatment desired, and the type of polynucleotide being administered. In general, a composition of the present invention includes from about 1 ng to about 30 mg of a polynucleotide, more preferably, from about 100 ng to about 10 mg of a polynucleotide.

Certain preferred compositions of the present invention may include about 1 ng of a polynucleotide, about 5 ng of a polynucleotide, about 10 ng of a polynucleotide, about 50 ng of a polynucleotide, about 100 ng of a polynucleotide, about 500 ng of a polynucleotide, about 1 μ g of a polynucleotide, about 5 μ g of a polynucleotide, about 10 μ g of a polynucleotide, about 50 μ g of a polynucleotide, about 100 μ g of a polynucleotide, about 150 μ g of a polynucleotide, about 200 μ g of a polynucleotide, about 250 μ g of a polynucleotide, about 300 μ g of a polynucleotide, about 350 μ g of a polynucleotide, about 400 μ g of a polynucleotide, about 450 μ g of a polynucleotide, about 500 μ g of a polynucleotide, about 550 μ g of a polynucleotide, about 600 μ g of a polynucleotide, about 650 μ g of a polynucleotide, about 700 μ g of a polynucleotide, about 750 μ g of a polynucleotide, about 800 μ g of a polynucleotide, about 850 μ g of a polynucleotide, about 900 μ g of a

polynucleotide, about 950 µg of a polynucleotide, about 1 mg of a polynucleotide, about 5 mg of a polynucleotide, about 10 mg of a polynucleotide, about 15 mg of a polynucleotide, about 20 mg of a polynucleotide, about 25 mg of a polynucleotide, and about 30 mg of a polynucleotide.

The choice of polynucleotide form depends in part on the desired kinetics and duration of expression. When long-term expression of the polypeptide encoded by the polynucleotide is desired, the preferred form is DNA, preferably plasmid DNA. Alternatively, when short-term expression of the polypeptide encoded by the polynucleotide is desired, the preferred form is RNA, preferably messenger RNA, since RNA is rapidly translated into polypeptide, but is degraded more quickly than DNA.

In one embodiment, a polynucleotide of the present invention is RNA. Preferably in this embodiment, the RNA is in the form of messenger RNA (mRNA). Methods for introducing RNA sequences into vertebrate cells is described in U.S. Patent No. 5,580,859, the disclosure of which is incorporated herein by reference in its entirety.

Alternatively, the RNA is in the form of an RNA virus genome. Preferably an RNA virus genome of the present invention is noninfectious, (*i.e.*, does not result in the production of infectious virus particles in vertebrate cells). Suitable RNA virus genomes include, but are not limited to, alphavirus genomes, picornavirus genomes, and retrovirus genomes. Methods for the *in vivo* introduction of non-infectious viral genomes to vertebrate tissues are well known to those of ordinary skill in the art and are described, *e.g.*, in Altman-Hamamdzcic, S., *et al.*, *Gene Therapy* 4, 815-822 (1997), in U.S. Patent No. 4,980,289, December 25, 1990, and in Miller, A.D., *et al.*, *Meth. Enzymol.* 217:581-599 (1993), the disclosures of which are incorporated herein by reference in their entireties. Viral replicons, *i.e.*, non-infectious RNA virus genomes packaged in a viral coat, *e.g.*, a picornavirus coat or an alphavirus coat, are also useful for efficient administration of RNA. *See, e.g.*, US Patent No. 5,766,602, U.S. Patent No. 5,614,413, and PCT Publication No.

WO 95/07994, the disclosures of which are incorporated herein by reference in their entireties.

Preferably, the polynucleotide is DNA. In the case of DNA, a polynucleotide encoding a polypeptide is normally operably associated with a promoter. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can be operably associated with the polynucleotide to direct cell-specific transcription.

An operable association is when a polynucleotide encoding a gene product, *e.g.*, a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the molecule under the influence or control of the regulatory sequence(s). Two DNA fragments (such as a polypeptide-coding polynucleotide and a promoter associated with the 5' end of the polynucleotide) are "operably associated" if induction of promoter function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the gene product, or (3) interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a polynucleotide encoding a polypeptide if the promoter was capable of effecting transcription of that polynucleotide.

A variety of transcription control regions are known to those skilled in the art. Preferred transcription control regions include those which function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (preferably the immediate early promoter, preferably in conjunction with intron-A), simian virus 40 (preferably the early promoter), retroviruses (such as Rous sarcoma virus), and picornaviruses (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence). Other preferred transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and

rabbit β -globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (*e.g.*, promoters inducible by interferons or interleukins).

Preferably, a DNA polynucleotide of the present invention is part of a circular or linearized plasmid which is preferably non-infectious (*i.e.*, does not result in the production of infectious virus particles in vertebrate cells), and nonintegrating (*i.e.*, does not integrate into the genome of vertebrate cells). A linearized plasmid is a plasmid that was previously circular but has been linearized, for example, by digestion with a restriction endonuclease.

Alternatively, DNA virus genomes may be used to administer DNA polynucleotides into vertebrate cells. Preferably a DNA virus genome of the present invention is noninfectious, (*i.e.*, does not result in the production of infectious virus particles in vertebrate cells), and nonintegrating (*i.e.*, does not integrate into the genome of vertebrate cells). Suitable DNA virus genomes include herpesvirus genomes, adenovirus genomes, adeno-associated virus genomes, and poxvirus genomes. References citing methods for the *in vivo* introduction of non-infectious virus genomes to vertebrate tissues are well known to those of ordinary skill in the art, and are cited *supra*.

Polynucleotides of the present invention may be associated with additional polynucleotides which encode secretory or signal peptides, which direct the secretion of the polypeptide encoded by the polynucleotide of the present invention. Those of ordinary skill in the art are aware that polypeptides secreted by vertebrate cells normally have a signal peptide which is cleaved from the complete polypeptide to produce a secreted "mature" form of the polypeptide.

Polypeptides

Compositions of the present invention may be used to deliver a wide variety of polypeptides to a vertebrate in need of any given polypeptide. Suitable polypeptides include, but are not limited to: therapeutic polypeptides, antigenic polypeptides, immunogenic polypeptides, immunomodulatory polypeptides, functional self polypeptides, and other functional polypeptides.

As used herein, a "therapeutic polypeptide" is a polypeptide which when delivered to a vertebrate, treats, *i.e.*, cures, ameliorates, or lessens the symptoms of, a given disease in that vertebrate, or alternatively, prolongs the life of the vertebrate by slowing the progress of a terminal disease. As used herein, an "immunomodulatory polypeptide" is a polypeptide which, when delivered to a vertebrate, can alter, enhance, suppress, or regulate an immune response in a vertebrate. Immunomodulatory polypeptides are a subset of therapeutic polypeptides. Therapeutic and immunomodulatory polypeptides of the present invention include, but are not limited to, cytokines, chemokines, lymphokines, ligands, receptors, hormones, apoptosis-inducing polypeptides, enzymes, antibodies, and growth factors. Examples include, but are not limited to granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), colony stimulating factor (CSF), interleukin 2 (IL-2), interleukin-3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), interleukin 15 (IL-15), interleukin 18 (IL-18), interferon alpha (IFN α), interferon beta (IFN β), interferon gamma (IFN γ), interferon omega (IFN ω), interferon tau (IFN τ), interferon gamma inducing factor I (IGIF), transforming growth factor beta (TGF- β), RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), macrophage inflammatory proteins (*e.g.*, MIP-1 alpha and MIP-1 beta), *Leishmania* elongation initiating factor (LEIF), platelet derived growth factor (PDGF), tumor necrosis factor (TNF), growth factors, *e.g.*, epidermal growth

factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor, (FGF), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-2 (NT-2), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), neurotrophin-5 (NT- 5), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), erythropoietin (EPO), and insulin.

Therapeutic polypeptides of the present invention may be used to treat diseases such as Parkinson's disease, cancer, and heart disease. In addition, therapeutic polypeptides may be used to treat autoimmune disorders such as multiple sclerosis; Sjogren's syndrome; sarcoidosis; insulin dependent diabetes mellitus; autoimmune thyroiditis; arthritis (*e.g.*), osteoarthritis, rheumatoid arthritis, reactive arthritis, and psoriatic arthritis; ankylosing spondylitis; and scleroderma. Also, therapeutic polypeptides of the present invention can be used to treat acute and chronic inflammatory disorders, to promote wound healing, and to prevent rejection after transplantation of cells, tissues, or organs.

Therapeutic polypeptides of the present invention, for example, neurotrophic factors (NTFs), may be used to promote the survival, maintenance, differentiation, repair, regeneration, and growth of cells in the brain, spinal cord, and peripheral nerves. Suitable NTFs include, but are not limited to, NGF, BDNF, the Neurotrophins or NTs such as NT-2, NT-3, NT-4, NT-5, GDNF, CNTF, as well as others. The administration of purified recombinant NTFs represents a clinical strategy for treatment of such acute and chronic nervous system disorders. Such disorders include, but are not limited to mechanical or chemical brain or spinal cord injury, Parkinson's Disease, Alzheimer's Disease and other dementias, Amyotrophic Lateral Sclerosis and Multiple Sclerosis.

Therapeutic polypeptides of the present invention, for example, growth factors, may be used to promote wound healing. Useful growth factors include, but are not limited to FGF, and EGF.

Therapeutic polypeptides of the present invention may be used to promote cell suicide (termed "apoptosis"). Suitable apoptotic polypeptides include the BAX protein. Alternatively, therapeutic polypeptides of the

present invention may be used to prevent apoptosis. Suitable apoptosis antagonists include the BAX antagonist Bcl-2. A disease which may be treated with apoptosis-inhibiting polypeptides is Muscular Dystrophy (MD), where patients have a defective protein called Dystrophin. Dystrophin is required for proper muscle function. The non-defective, normal Dystrophin may act as an antigen if delivered via plasmid DNA to patients with MD. In this case, muscle cells transduced with DNA encoding normal Dystrophin would be recognized by the immune system and killed by Dystrophin-specific T cell based responses. Such T cell based killing is known to kill cells by inducing apoptosis. If the normal, and potentially immunogenic, Dystrophin could be delivered into muscle cells along with Bcl-2 or other apoptosis-preventing protein, one would expect that CTL would be unable to kill the muscle cells. This reasoning applies to many genetic diseases where treatment involves delivery of a "normal", and therefore potentially immunogenic, copy of a protein.

As used herein, a "functional self polypeptide" is a polypeptide which is required for normal functioning of a vertebrate, but because of, *e.g.*, genetic disease, cancer, environmental damage, or other cause, is missing, defective, or non-functional in a given individual. A composition of the present invention is used to restore the individual to a normal state by supplying the necessary polypeptide. Examples of functional self polypeptides include insulin, dystrophin, cystic fibrosis transmembrane conductance regulator, granulocyte macrophage colony stimulating factor, granulocyte colony stimulating factor, macrophage colony stimulating factor colony stimulating factor, interleukin 2, interleukin-3, interleukin 4, interleukin 5, interleukin 6, interleukin 7, interleukin 8, interleukin 10, interleukin 12, interleukin 15, interleukin 18, interferon alpha, interferon beta, interferon gamma, interferon omega, interferon tau, interferon gamma inducing factor I, transforming growth factor beta, RANTES, Flt-3 ligand, macrophage inflammatory proteins, platelet derived growth factor, tumor necrosis factor, epidermal growth factor, vascular epithelial growth factor, fibroblast growth factor, insulin-like growth factors I and II, insulin-like growth factor binding proteins,

nerve growth factor, brain derived neurotrophic factor, neurotrophin-2, neurotrophin-3, neurotrophin-4, neurotrophin-5, glial cell line-derived neurotrophic factor, ciliary neurotrophic factor, and erythropoietin. Examples of diseases or disorders that may be treated with functional self polypeptides include, but are not limited to: diabetes, muscular dystrophy, multiple sclerosis, Parkinson's disease, Alzheimer's disease, arthritis, sickle cell anemia, and hemophilia.

As used herein, an antigenic polypeptide or an immunogenic polypeptide is a polypeptide which, when introduced into a vertebrate, reacts with the immune system molecules of the vertebrate, *i.e.*, is antigenic, and/or induces an immune response in the vertebrate, *i.e.*, is immunogenic. It is quite likely that an immunogenic polypeptide will also be antigenic, but an antigenic polypeptide, because of its size or conformation, may not necessarily be immunogenic. Examples of antigenic and immunogenic polypeptides include, but are not limited to, polypeptides from infectious agents such as bacteria, viruses, parasites, or fungi, allergens such as those from pet dander, plants, dust, and other environmental sources, as well as certain self polypeptides, for example, tumor-associated antigens.

Antigenic and immunogenic polypeptides of the present invention can be used to prevent or treat, *i.e.*, cure, ameliorate, lessen the severity of, or prevent or reduce contagion of viral, bacterial, fungal, and parasitic infectious diseases, as well as to treat allergies.

In addition, antigenic and immunogenic polypeptides of the present invention can be used to prevent or treat, *i.e.*, cure, ameliorate, or lessen the severity of cancer including, but not limited to, cancers of oral cavity and pharynx (*i.e.*, tongue, mouth, pharynx), digestive system (*i.e.*, esophagus, stomach, small intestine, colon, rectum, anus, anal canal, anorectum, liver, gallbladder, pancreas), respiratory system (*i.e.*, larynx, lung), bones, joints, soft tissues (including heart), skin, melanoma, breast, reproductive organs (*i.e.*, cervix, endometrium, ovary, vulva, vagina, prostate, testis, penis), urinary system (*i.e.*, urinary bladder, kidney, ureter, and other urinary organs), eye, brain, endocrine system (*i.e.*, thyroid and other endocrine), lymphoma (*i.e.*,

hodgkin's disease, non-hodgkin's lymphoma), multiple myeloma, leukemia (i.e., acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia, chronic myeloid leukemia).

Examples of viral antigenic and immunogenic polypeptides include, but are not limited to, adenovirus polypeptides, alphavirus polypeptides, calicivirus polypeptides, e.g., a calicivirus capsid antigen, coronavirus polypeptides, distemper virus polypeptides, Ebola virus polypeptides, enterovirus polypeptides, flavivirus polypeptides, hepatitis virus (AE) polypeptides, e.g., a hepatitis B core or surface antigen, herpesvirus polypeptides, e.g., a herpes simplex virus or varicella zoster virus glycoprotein, immunodeficiency virus polypeptides, e.g., the human immunodeficiency virus envelope or protease, infectious peritonitis virus polypeptides, influenza virus polypeptides, e.g., an influenza A hemagglutinin, neuraminidase, or nucleoprotein, leukemia virus polypeptides, Marburg virus polypeptides, orthomyxovirus polypeptides, papilloma virus polypeptides, parainfluenza virus polypeptides, e.g., the hemagglutinin/neuraminidase, paramyxovirus polypeptides, parvovirus polypeptides, pestivirus polypeptides, picorna virus polypeptides, e.g., a poliovirus capsid polypeptide, pox virus polypeptides, e.g., a vaccinia virus polypeptide, rabies virus polypeptides, e.g., a rabies virus glycoprotein G, reovirus polypeptides, retrovirus polypeptides, and rotavirus polypeptides.

Examples of bacterial antigenic and immunogenic polypeptides include, but are not limited to, *Actinomyces* polypeptides, *Bacillus* polypeptides, *Bacteroides* polypeptides, *Bordetella* polypeptides, *Bartonella* polypeptides, *Borrelia* polypeptides, e.g., *B. burgdorferi* OspA, *Brucella* polypeptides, *Campylobacter* polypeptides, *Capnocytophaga* polypeptides, *Chlamydia* polypeptides, *Clostridium* polypeptides, *Corynebacterium* polypeptides, *Coxiella* polypeptides, *Dermatophilus* polypeptides, *Enterococcus* polypeptides, *Ehrlichia* polypeptides, *Escherichia* polypeptides, *Francisella* polypeptides, *Fusobacterium* polypeptides, *Haemobartonella* polypeptides, *Haemophilus* polypeptides, e.g., *H. influenzae* type b outer membrane protein, *Helicobacter* polypeptides, *Klebsiella* polypeptides,

L-form bacteria polypeptides, *Leptospira* polypeptides, *Listeria* polypeptides, *Mycobacteria* polypeptides, *Mycoplasma* polypeptides, *Neisseria* polypeptides, *Neorickettsia* polypeptides, *Nocardia* polypeptides, *Pasteurella* polypeptides, *Peptococcus* polypeptides, *Peptostreptococcus* polypeptides, *Pneumococcus* polypeptides, *Proteus* polypeptides, *Pseudomonas* polypeptides, *Rickettsia* polypeptides, *Rochalimaea* polypeptides, *Salmonella* polypeptides, *Shigella* polypeptides, *Staphylococcus* polypeptides, *Streptococcus* polypeptides, e.g., *S. pyogenes* M proteins, *Treponema* polypeptides, and *Yersinia* polypeptides, e.g., *Y. pestis* F1 and V antigens.

Examples of fungal immunogenic and antigenic polypeptides include, but are not limited to, *Absidia* polypeptides, *Acremonium* polypeptides, *Alternaria* polypeptides, *Aspergillus* polypeptides, *Basidiobolus* polypeptides, *Bipolaris* polypeptides, *Blastomyces* polypeptides, *Candida* polypeptides, *Coccidioides* polypeptides, *Conidiobolus* polypeptides, *Cryptococcus* polypeptides, *Curvalaria* polypeptides, *Epidermophyton* polypeptides, *Exophiala* polypeptides, *Geotrichum* polypeptides, *Histoplasma* polypeptides, *Madurella* polypeptides, *Malassezia* polypeptides, *Microsporum* polypeptides, *Moniliella* polypeptides, *Mortierella* polypeptides, *Mucor* polypeptides, *Paecilomyces* polypeptides, *Penicillium* polypeptides, *Phialemonium* polypeptides, *Phialophora* polypeptides, *Prototheca* polypeptides, *Pseudallescheria* polypeptides, *Pseudomicrodochium* polypeptides, *Pythium* polypeptides, *Rhinosporidium* polypeptides, *Rhizopus* polypeptides, *Scolecobasidium* polypeptides, *Sporothrix* polypeptides, *Stemphylium* polypeptides, *Trichophyton* polypeptides, *Trichosporon* polypeptides, and *Xylohypha* polypeptides.

Examples of protozoan parasite immunogenic and antigenic polypeptides include, but are not limited to, *Babesia* polypeptides, *Balantidium* polypeptides, *Besnoitia* polypeptides, *Cryptosporidium* polypeptides, *Eimeria* polypeptides, *Encephalitozoon* polypeptides, *Entamoeba* polypeptides, *Giardia* polypeptides, *Hammondia* polypeptides, *Hepatozoon* polypeptides, *Isospora* polypeptides, *Leishmania* polypeptides, *Microsporidia* polypeptides, *Neospora* polypeptides, *Nosema* polypeptides,

Pentatrichomonas polypeptides, *Plasmodium* polypeptides, e.g., *P. falciparum* circumsporozoite (PfCSP), sporozoite surface protein 2 (PfSSP2), carboxyl terminus of liver stage antigen 1 (PfLSA1 c-term), and exported protein 1 (PfExp-1), *Pneumocystis* polypeptides, *Sarcocystis* polypeptides, *Schistosoma* polypeptides, *Theileria* polypeptides, *Toxoplasma* polypeptides, and *Trypanosoma* polypeptides.

Examples of helminth parasite immunogenic and antigenic polypeptides include, but are not limited to, *Acanthocheilonema* polypeptides, *Aelurostrongylus* polypeptides, *Ancylostoma* polypeptides, *Angiostrongylus* polypeptides, *Ascaris* polypeptides, *Brugia* polypeptides, *Bunostomum* polypeptides, *Capillaria* polypeptides, *Chabertia* polypeptides, *Cooperia* polypeptides, *Crenosoma* polypeptides, *Dictyocaulus* polypeptides, *Dioctophyme* polypeptides, *Dipetalonema* polypeptides, *Diphyllbothrium* polypeptides, *Diplydium* polypeptides, *Dirofilaria* polypeptides, *Dracunculus* polypeptides, *Enterobius* polypeptides, *Filaroides* polypeptides, *Haemonchus* polypeptides, *Lagochilascaris* polypeptides, *Loa* polypeptides, *Mansonella* polypeptides, *Muellerius* polypeptides, *Nanophyetus* polypeptides, *Necator* polypeptides, *Nematodirus* polypeptides, *Oesophagostomum* polypeptides, *Onchocerca* polypeptides, *Opisthorchis* polypeptides, *Ostertagia* polypeptides, *Parafilaria* polypeptides, *Paragonimus* polypeptides, *Parascaris* polypeptides, *Physaloptera* polypeptides, *Protostrongylus* polypeptides, *Setaria* polypeptides, *Spirocerca* polypeptides, *Spirometra* polypeptides, *Stephanofilaria* polypeptides, *Strongyloides* polypeptides, *Strongylus* polypeptides, *Thelazia* polypeptides, *Toxascaris* polypeptides, *Toxocara* polypeptides, *Trichinella* polypeptides, *Trichostrongylus* polypeptides, *Trichuris* polypeptides, *Uncinaria* polypeptides, and *Wuchereria* polypeptides.

Examples of ectoparasite immunogenic and antigenic polypeptides include, but are not limited to, polypeptides (including protective antigens as well as allergens) from fleas; ticks, including hard ticks and soft ticks; flies, such as midges, mosquitos, sand flies, black flies, horse flies, horn flies, deer

flies, tsetse flies, stable flies, myiasis-causing flies and biting gnats; ants; spiders, lice; mites; and true bugs, such as bed bugs and kissing bugs.

5 Examples of tumor-associated antigenic and immunogenic polypeptides include, but are not limited to, tumor-specific immunoglobulin variable regions (*e.g.*, B cell lymphoma idiotypes), GM2, Tn, sTn, Thompson-Friedenreich antigen (TF), Globo H, Le(y), MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, carcinoembryonic antigens, beta chain of human chorionic gonadotropin (hCG beta), HER2/neu, PSMA, EGFRvIII, KSA, PSA, PSCA, GP100, MAGE 1, MAGE 2, TRP 1, TRP 2, tyrosinase, MART-1, PAP, CEA, BAGE, MAGE, RAGE, and related proteins.

10 Also included as polypeptides of the present invention are fragments, derivatives, analogs, or variants of the foregoing polypeptides, and any combination of the foregoing polypeptides. Additional polypeptides may be found, for example in "*Foundations in Microbiology*," Talaro, *et al.*, eds., McGraw-Hill Companies (Oct.,1998), Fields, *et al.*, "*Virology*," 3d ed., 15 Lippincott-Raven (1996), "*Biochemistry and Molecular Biology of Parasites*," Marr, *et al.*, eds., Academic Press (1995), and Deacon, J., "*Modern Mycology*," Blackwell Science Inc (1997), which are incorporated herein by reference.

Transfection Facilitating Materials

Compositions of the present invention can also include one or more transfection facilitating materials that facilitate delivery of polynucleotides to the interior of a cell, and/or to a desired location within a cell. Examples of the transfection facilitating materials include, but are not limited to lipids, preferably cationic lipids; inorganic materials such as calcium phosphate, alum (aluminum sulfate), and metal (*e.g.*, gold or tungsten) particles (*e.g.*, "powder" type delivery solutions); peptides, including cationic peptides, targeting peptides for selective delivery to certain cells or intracellular organelles such as the nucleus or nucleolus, and amphipathic peptides, *i.e.* helix forming or pore forming peptides; basic proteins, such as histones; asialoproteins; viral proteins (*e.g.*, Sendai virus coat protein); pore-forming proteins; and polymers, including dendrimers, star-polymers, "homogenous" poly-amino acids (*e.g.*, poly-lysine, poly-arginine), "heterogenous" poly-amino acids (*e.g.*, mixtures of lysine & glycine), co-polymers, polyvinylpyrrolidinone (PVP), and polyethylene glycol (PEG). Furthermore, those auxiliary agents of the present invention which facilitate and enhance the entry of a polynucleotide into vertebrate cells *in vivo*, may also be considered "transfection facilitating materials."

Certain embodiments of the present invention may include lipids as a transfection facilitating material, including cationic lipids (*e.g.*, DMRIE, DOSPA, DC-Chol, GAP-DLRIE), basic lipids (*e.g.*, steryl amine), neutral lipids (*e.g.*, cholesterol), anionic lipids (*e.g.*, phosphatidyl serine), and zwitterionic lipids (*e.g.*, DOPE, DOPC), as described above. However, certain compositions and methods of the present invention, *e.g.*, those including or utilizing compositions comprising a sodium phosphate dissolved in an aqueous solution at a molar concentration from about 20 mM to about 300 mM, are preferably substantially free of cationic lipids.

Certain other compositions and methods of the present invention, *e.g.*, those including or utilizing compositions comprising a sodium phosphate dissolved in aqueous solution at a molar concentration from about 0.1 mM to

about 150 mM, where the aqueous solution is substantially free of chloride anion, always include cationic lipids as transfection facilitating agents. While not being bound by theory, cationic lipids are believed to bind effectively to negatively charged polynucleotides, thereby facilitating entry of the polynucleotide into cells. The use of cationic lipids is especially effective in the delivery of polynucleotides to non-muscle tissues, *e.g.*, pulmonary tissues, tumor tissues, skin, peritoneum, tissues of digestive system, or vascular tissues.

In the embodiments including cationic lipids, the polynucleotide construct(s) are combined with lipids by mixing, for example, a plasmid DNA solution and a solution of cationic lipid:co-lipid liposomes. Preferably, the concentration of each of the constituent solutions is adjusted prior to mixing such that the desired final plasmid DNA/cationic lipid:co-lipid ratio and the desired plasmid DNA final concentration will be obtained upon mixing the two solutions. For example, if the desired final solution is to be 150 mM sodium phosphate, the various components of the composition, *e.g.*, plasmid DNA, cationic lipid:co-lipid liposomes, and any other desired auxiliary agents, transfection facilitating materials, or additives are each prepared in 150 mM sodium phosphate and then simply mixed to afford the desired complex.

Alternatively, if the desired final solution is to be, *e.g.*, 150 mM sodium phosphate, certain components of the composition, *e.g.*, the auxiliary agent and/or cationic lipid:co-lipid liposomes, is prepared in a volume of water which is less than that of the final volume of the composition, and certain other components of the composition, *e.g.*, the plasmid DNA, is prepared in a solution of sodium phosphate at a higher concentration than 150 mM. In a volume such that when the components in water are added to the components in the sodium phosphate solution, the final composition is in an aqueous solution of 150 mM sodium phosphate. For example, the plasmid DNA could be prepared in 300 mM sodium phosphate at one half the final volume, the auxiliary agent and/or cationic lipid:co-lipid liposome is prepared in water at one half the final volume, and then these two elements are mixed together to produce the final composition.

The cationic lipid:co-lipid liposomes are preferably prepared by hydrating a thin film of the mixed lipid materials in an appropriate volume of aqueous solvent by vortex mixing at ambient temperatures for about 1 minute. The thin films are prepared by admixing chloroform solutions of the individual components to afford a desired molar solute ratio followed by aliquoting the desired volume of the solutions into a suitable container. The solvent is removed by evaporation, first with a stream of dry, inert gas (e.g. argon) followed by high vacuum treatment.

A transfection facilitating material can be used alone or in combination with one or more other transfection facilitating materials. Two or more transfection facilitating materials can be combined by chemical bonding (e.g., covalent and ionic such as in lipidated polylysine, PEGylated polylysine) (Toncheva, V., *et al.*, *Biochim. Biophys. Acta* 1380(3):354-368 (1998)), mechanical mixing (e.g., free moving materials in liquid or solid phase such as "polylysine + cationic lipids") (Gao, X., and Huang, L., *Biochemistry* 35:1027-1036 (1996); Trubetskoy, V.S., *et al.*, *Biochem. Biophys. Acta* 1131:311-313 (1992)), and aggregation (e.g., co-precipitation, gel forming such as in cationic lipids + poly-lactide co-galactide, and polylysine + gelatin).

Other Additives

Other hydrophobic and amphiphilic additives, such as, for example, sterols, fatty acids, gangliosides, glycolipids, lipopeptides, liposaccharides, neobees, niosomes, prostaglandins and sphingolipids, may also be included in the compositions of the present invention. In such compositions, these additives may be included in an amount between about 0.1 mol % and about 99.9 mol % (relative to total lipid). Preferably, these additives comprise about 1-50 mol % and, most preferably, about 2-25 mol %. Preferred additives include lipopeptides, liposaccharides and steroids.

Methods and Administration

The present invention further provides methods for delivering a polypeptide into a vertebrate, which comprise administering to the vertebrate a composition as described herein; such that upon administration of the composition, the polypeptide is expressed in the vertebrate, in an amount sufficient to be detectable. Methods to detect polypeptides expressed in a vertebrate are well known to those of ordinary skill in the art and include, but are not limited to, serological methods to detect the polypeptide in serum, *e.g.*, western blotting, staining tissue sections by immunohistochemical methods, measuring an immune response generated by the vertebrate against the polypeptide, and measuring the activity of the polypeptide. Certain of these methods are disclosed in the Examples, below.

The present invention further provides a method for delivering a therapeutic polypeptide into a vertebrate, comprising administering to a vertebrate in need of the therapeutic polypeptide a composition as described herein. In this method, the composition comprises a polynucleotide encoding a therapeutic polypeptide. Upon administration of the composition according to this method, the needed therapeutic polypeptide is expressed in the vertebrate in a therapeutically effective amount.

Similarly, the present invention provides a method of enhancing or modulating an immune response in a vertebrate in need of such an enhanced or modulated immune response, comprising administering to the vertebrate a composition as described herein. In this method, the composition contains a polynucleotide encoding an immunogenic and/or immunomodulatory polypeptide. Upon administration of the composition according to this method, the needed immunogenic and/or immunomodulatory polypeptide is expressed in the vertebrate, in a sufficient amount to induce and/or modify a desired immune response in the vertebrate to prevent disease, cure disease, reduce the severity of disease symptoms, or prolong the life of the vertebrate.

Also, the present invention provides a method of enhancing or modulating an immune response in a healthy vertebrate for large-scale

antibody production, comprising administering to the vertebrate a composition as described herein. In this method, the composition contains a polynucleotide encoding an immunogenic and/or immunomodulatory polypeptide. Upon administration of the composition according to this method, the immunogenic and/or immunomodulatory polypeptide is expressed in the vertebrate, in a sufficient amount to produce a vigorous antibody response in the vertebrate. The antibodies thus produced are then recovered from the vertebrate by, for example, the collection of serum, milk, or saliva. Such antibodies may be useful for research or diagnostic purposes, or for additional therapies in vertebrates in need of such therapies. For example, passive antibody treatment using antibodies produced by this method may prevent disease, cure disease, reduce the severity of disease symptoms, or prolong the life of a vertebrate.

Moreover, the present invention further provides a method of delivering a physiologically or metabolically necessary polypeptide to a vertebrate incapable of making a functional form of the polypeptide, comprising administering to the vertebrate a composition as disclosed herein. According to this method, the composition contains a polynucleotide encoding a functional self polypeptide. Upon administration of the composition according to this method, the needed functional self polypeptide is expressed in the vertebrate, in a sufficient amount to supply the vertebrate's requirements for the polypeptide.

An important aspect of the present invention is that use of the claimed compositions in any of the above methods allows the skilled artisan to reduce the amount of polynucleotide included in the composition relative to methods utilizing existing compositions, *e.g.*, those which formulate the polynucleotide in saline or water. Even though the amount of polynucleotide is reduced, sufficient protein expression occurs in the treated vertebrate. Such a reduction in polynucleotide will significantly reduce the cost of producing compositions of the present invention. Accordingly, one embodiment of the present invention is a method to reduce the amount of polynucleotide required to obtain a desired clinical response in a vertebrate, comprising administering to the vertebrate a composition as disclosed herein.

In any of the methods disclosed herein, it is preferred that the composition be delivered to a mammal. More preferably, the mammal is a human.

Administration of the compositions of the present invention according to any of the above methods can be accomplished according to any of various methods known in the art. For example, U.S. Patent No. 5,676,954, incorporated herein by reference in its entirety, reports on the injection of genetic material, complexed with cationic lipid carriers, into mice. Also, U.S. Patent Nos. 5,589,466, 5,693,622, 5,580,859, 5,703,055, and PCT international patent application PCT/US94/06069 (WO 94/29469), the disclosures of which are incorporated herein by reference in their entireties, provide methods for delivering compositions comprising naked DNA, or DNA cationic lipid complexes to vertebrates.

More specifically, the compositions of the present invention may be administered to any tissue of a vertebrate, including, but not limited to, muscle, skin, brain tissue, lung tissue, liver tissue, spleen tissue, bone marrow tissue, thymus tissue, heart tissue, *e.g.*, myocardium, endocardium, and pericardium, lymph tissue, blood tissue, bone tissue, pancreas tissue, kidney tissue, gall bladder tissue, stomach tissue, intestinal tissue, testicular tissue, ovarian tissue, uterine tissue, vaginal tissue, rectal tissue, nervous system tissue, eye tissue, glandular tissue, tongue tissue, and connective tissue, *e.g.*, cartilage.

Furthermore, the compositions of the present invention may be administered to any internal cavity of a vertebrate, including, but not limited to, the lungs, the mouth, the nasal cavity, the stomach, the peritoneal cavity, the intestine, any heart chamber, veins, arteries, capillaries, lymphatic cavities, the uterine cavity, the vaginal cavity, the rectal cavity, joint cavities, ventricles in brain, spinal canal in spinal cord, and the ocular cavities.

Preferably, the compositions comprising sodium phosphate dissolved in aqueous solution at a molar concentration from about 20 mM to about 300 mM are delivered to muscle, either skeletal muscle or cardiac muscle, and those embodiments comprising sodium phosphate dissolved in aqueous

solution at a molar concentration from about 0.1 mM to about 150 mM and a cationic lipid are administered to lung tissue. Preferred modes for administration to lung tissue are disclosed in Wheeler, C.J., *et al.*, *Proc. Natl. Acad. Sci. USA* 93:11454-11459 (1996), which is incorporated herein by reference in its entirety.

According to the disclosed methods, compositions of the present invention are preferably administered by intramuscular (i.m.), subcutaneous (s.c.), or intrapulmonary routes. Other suitable routes of administration include intratracheal, transdermal, interdermal, intraocular, intranasal, inhalation, transmucosal (*i.e.*, across a mucous membrane), intracavity (*e.g.*, oral, vaginal, rectal, nasal, peritoneal, ventricular, or intestinal), and intravenous (i.v.) administration.

Any mode of administration can be used so long as the mode results in the expression of the desired peptide or protein, in the desired tissue, in an amount sufficient to be detectable, and/or prophylactically or therapeutically effective. Administration means of the present invention include needle injection, catheter infusion, biolistic injectors, particle accelerators (*e.g.*, "gene guns" or pneumatic "needleless" injectors) Med-E-Jet (Vahlsing, H., *et al.*, *J. Immunol. Methods* 171,11-22 (1994)), Pigjet (Schrijver, R., *et al.*, *Vaccine* 15, 1908-1916 (1997)), Biojector (Davis, H., *et al.*, *Vaccine* 12, 1503-1509 (1994); Gramzinski, R., *et al.*, *Mol. Med.* 4, 109-118 (1998)), AdvantaJet (Linmayer, I., *et al.*, *Diabetes Care* 9:294-297 (1986)), Medi-jector (Martins, J., and Roedl, E. *J. Occup. Med.* 21:821-824 (1979)), gelfoam sponge depots, other commercially available depot materials (*e.g.*, hydrogels), osmotic pumps (*e.g.*, Alza minipumps), oral or suppositorial solid (tablet or pill) pharmaceutical formulations, topical skin creams, and decanting, use of polynucleotide coated suture (Qin, Y., *et al.*, *Life Sciences* 65, 2193-2203 (1999)) or topical applications during surgery. The preferred modes of administration are intramuscular needle-based injection and pulmonary application via catheter infusion. Each of the references cited in this paragraph is incorporated herein by reference in its entirety.

Determining an effective amount of a composition depends upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the subject, the precise condition requiring treatment and its severity, and the route of administration. Based on the above factors, determining the precise amount, number of doses, and timing of doses are within the ordinary skill in the art and will be readily determined by the attending physician or veterinarian.

Compositions of the present invention can be formulated according to known methods. Suitable preparation methods are described, for example, in *Remington's Pharmaceutical Sciences*, 16th Edition, A. Osol, ed., Mack Publishing Co., Easton, PA (1980), and *Remington's Pharmaceutical Sciences*, 19th Edition, A.R. Gennaro, ed., Mack Publishing Co., Easton, PA (1995), both of which are incorporated herein by reference in their entireties. Although the composition is preferably administered as an aqueous solution, it can be formulated as an emulsion, gel, solution, suspension, lyophilized form, or any other form known in the art. According to the present invention, if the composition is formulated other than as an aqueous solution, it will require resuspension in an aqueous solution prior to administration. In addition, the composition may contain pharmaceutically acceptable additives including, for example, diluents, binders, stabilizers, and preservatives.

For aqueous compositions used *in vivo*, the use of sterile pyrogen-free water is preferred. Such formulations will contain an effective amount of a polynucleotide together with a suitable amount of an aqueous solution in order to prepare pharmaceutically acceptable compositions suitable for administration to a vertebrate

Pharmaceutical Kits

The present invention also provides kits for use in delivering a polypeptide to a vertebrate. Each kit includes a container holding about 1 ng to about 30 mg of a polynucleotide which operably encodes a polypeptide

within vertebrate cells *in vivo*. Furthermore, each kit includes either (a) an amount of sodium phosphate which, when dissolved in a prescribed volume of distilled water, results in an aqueous solution with a molar concentration of said salt from about 20 mM to about 300 mM, and reaction, association, or dissociation products thereof; and optionally, an administration means; whereby the polynucleotide is provided in a prophylactically or therapeutically effective amount to treat a vertebrate; or (b) an amount of sodium phosphate which, when dissolved in an prescribed volume of distilled water, results in an aqueous solution with a molar concentration of said salt from about 0.1 mM to about 150 mM, and reaction, association, or dissociation products thereof, and where the aqueous solution formed thereby is essentially free of chloride anion; a cationic lipid; and optionally, an administration means; whereby the polynucleotide is provided in a prophylactically or therapeutically effective amount.. Any of the components of pharmaceutical kits (a) or (b) can be provided in a single container or in multiple containers. Preferably, the kit includes from about 1 ng to about 30 mg of a polynucleotide, more preferably, the kit includes from about 100 ng to about 10 mg of a polynucleotide.

Any suitable container or containers may be used with pharmaceutical kits. Examples of containers include, but are not limited to, glass containers, plastic containers, or strips of plastic or paper.

Each of the pharmaceutical kits may further comprise an administration means. Means for administration include, but are not limited to syringes and needles, catheters, biolistic injectors, particle accelerators, *i.e.*, "gene guns," pneumatic "needleless" injectors, gelfoam sponge depots, other commercially available depot materials, *e.g.*, hydrogels, osmotic pumps, and decanting, polynucleotide coated sutures, skin patches, or topical applications during surgery.

The kit can further comprise an instruction sheet for administration of the composition to a vertebrate. The polynucleotide components of the composition are preferably provided as a liquid solution or they may be provided in lyophilized form as a dried powder or a cake. If the polynucleotide is provided in lyophilized form, the dried powder or cake may

also include any salts, auxiliary agents, transfection facilitating agents, and additives of the composition in dried form. Such a kit may further comprise a container with an exact amount of sterile pyrogen-free water, for precise reconstitution of the lyophilized components of the composition.

The container in which the composition is packaged prior to use can comprise a hermetically sealed container enclosing an amount of the lyophilized formulation or a solution containing the formulation suitable for a pharmaceutically effective dose thereof, or multiples of an effective dose. The composition is packaged in a sterile container, and the hermetically sealed container is designed to preserve sterility of the pharmaceutical formulation until use. Optionally, the container can be associated with administration means and/or instruction for use.

Examples

Having now generally described the invention, the same will become more readily understood by reference to the following specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

Described herein are: 1) the *in vitro* characterization of biological activities of and IFNs delivered by plasmid DNA (anti-proliferative activity and anti-viral activity *in vitro*); 2) *in vivo* expression of cytokines following *in vivo* administration of cytokine-expressing pDNA; and 3) the *in vivo* characterization of anti-tumor activity of cytokines in murine models of solid and metastatic tumors following intratumoral, intramuscular or intra-cavity administration of cytokine-encoding pDNA.

The cytokine-encoding polynucleotide constructs have potent anti-proliferative activity *in vitro*. Moreover, the *in vivo* anti-tumor activities of IFN ω , IFN α , IL-2, and IL-12 are herein demonstrated in multiple murine tumor models including nude mice bearing subcutaneous human tumors, or in

immunocompetent mice bearing murine solid and metastatic tumors. Intratumoral, intramuscular, or intraperitoneal injection of the cytokine-encoding plasmids is shown to result in a statistically significant slowing of tumor growth and/or a statistically significant increase in survival. In addition to the potent antitumor effects of the cytokine plasmids delivered via intratumoral or intramuscular injection, this is the first *in vivo* demonstration of anti-tumor activity for human interferon- ω . Moreover, the *in vivo* antitumor activity of IL-2 in the treatment of peritoneally disseminated cancers, such as ovarian metastatic melanoma is also demonstrated.

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Example 1

Construction of Expression Vectors

5 Three basic eukaryotic expression plasmid vectors, termed VR1012, VR1055 and VR1033 were used in the construction of all plasmids used in the following examples. The blank plasmids, VR1012 and VR1055 differ only in transcriptional termination sequences. The backbone of both plasmids is derived from pUC19, with the beta-lactamase (ampicillin resistance) gene replaced by the aminoglycoside acetyltransferase (kanamycin resistance) gene
10 from pET9a (Novagen, Madison, WI). Both plasmids direct eukaryotic gene expression from a cassette containing the human cytomegalovirus immediate early I (CMV IE) gene promoter/enhancer, CMV IE 5' untranslated (UT) sequence, and intron A. Following these regulatory elements is a cloning polylinker for insertion of polypeptide coding sequences. Following the polylinker in VR1012 is the 3' UT sequence from the bovine growth hormone gene for polyadenylation and transcriptional termination. In VR1055, the transcriptional terminator region includes a polyadenylation and termination signals derived from the rabbit b-globin gene. VR1033 is identical to VR1012,
15 except that it contains a cap-independent translational enhancer from the encephalomyocarditis virus within the cloning polylinker sequence. This sequence allows the production of two different polypeptides from a single expressed mRNA.

Plasmid VR4101 (murine interferon α (mIFN α)) was constructed by
25 cloning the murine interferon α cDNA into the vector VR1012 vector. The cDNA was obtained by amplifying the coding sequence from the plasmid RSV-"1 (Kelly, K.A. and P.M. Pitha, *Nucl. Acids Res.* 13: 805-823 (1985); Kelly, K.A. and P.M. Pitha, *Nucl. Acids Res.* 13: 825-839 (1985)), which was provided by Dr. Paula Pitha-Rowe of Johns Hopkins University. Plasmid
30 VR4111 was constructed by transferring the coding sequences from VR4101 to the VR1055 cloning vector. The oligonucleotide primers used for polymerase chain reaction (PCR) were 5'-AACTGCAGATGGCTAGGCTCTGTGCT-3' (SEQ ID No. 15) and 5'-

GAAG-ATCTTCATTTCTCTTCTC-TCAG-3' (SEQ ID No. 16). Reaction conditions were 30 cycles of 94°C for 1 minute (denaturing), 58°C for 2 minutes (annealing), and 72°C for 1 minute (extension).

5 Plasmid VR4102 (human interferon α (hIFN α)) was constructed by cloning the human interferon α cDNA into the VR1012 vector. The cDNA was obtained by amplifying the coding sequence from human genomic DNA prepared from a fresh blood sample. Plasmid VR4112 was constructed by transferring the coding sequence sequences from VR4102 to the VR1055 cloning vector. Genomic DNA was isolated using the QIAamp Blood Kit
10 (Qiagen, Inc.). The oligonucleotide primers used for PCR were 5'-AACTGCAGATGGCCTC-GCCCTTTGCT-3' (SEQ ID No. 17) and 5'-CGGGATCCTTATTCCTTC-CTCCTTAATC-3' (SEQ ID No. 18). Reaction conditions were 30 cycles of 94°C for 1 minute (denaturing), 58°C for 2 minutes (annealing), and 72°C for 1 minute (extension).

15 Plasmid VR4150 (human interferon ω (hIFN ω)) was constructed by cloning the human IFN ω cDNA into the VR1012 cloning vector. The cDNA was obtained by amplifying the coding sequence from human genomic DNA prepared from a fresh blood sample. Plasmid VR4151 (SEQ ID No. 1) was constructed by transferring the coding sequences from VR4150 to the VR1055 cloning vector. The oligonucleotide primers used for PCR were 5'-GCTCTAGATGGCCCTCCTGTTCCCT-3' (SEQ ID No. 19) and 5'-GCGG-ATCCTCAAGATGAGCCAGGTC-3' (SEQ ID No. 20). Reaction conditions were 30 cycles of 94°C for 1 minute (denaturing), 58°C for 2 minutes (annealing), and 72°C for 1 minute (extension).

25 Plasmid VR1110 (murine interleukin-2 (mIL-2)) was constructed by cloning modified murine IL-2 cDNA into the VR1012 vector. The 5' UT sequence and the two amino acids of the leader peptide were replaced with the rat insulin II gene 5' UT sequence and coding region of the first six amino acids of the rat preproinsulin leader peptide. The IL-2 cDNA was then cloned
30 into the BamHI site of VR1012.

Plasmid VR1103 (human interleukin-2 (hIL-2)) is identical to VR1110 with the exception that the murine IL-2 cDNA was replaced with the cDNA for human IL-2 (Parker et. al. 1996).

Plasmid VR4001 (murine interleukin-12 (mIL-12)) was constructed by cloning the cDNA's encoding the two murine subunits p35 and p40 into the VR1033 vector. Both cDNA's were obtained by amplifying the coding sequences from plasmids provided by Dr. Thomas Gajewski of The University of Chicago (J. Immun., 154:5637; J. Immun., 156:1095). The oligonucleotides used for PCR of p35 were 5'- CAT GCC ATG GGT CAA TCA CGC TAC CTC CTC TTT TTG G-3' (SEQ ID No. 23) and 5'- GCG GAT CCT CAG GCG GAG CTC AGA TAG CCC-3' (SEQ ID No. 24). The oligonucleotides used for PCR of p40 were 5'- ACG CGT CGA CAT GTG TCC TCA GAA GCT AAC CAT CTC-3' (SEQ ID No. 21) and 5'- GCG GAT CCC TAG GAT CGG ACC CTG CAG GGA ACA C-3' (SEQ ID No. 22). Reaction conditions were 30 cycles of 94°C for 1 minute (denaturing), 58°C for 2 minutes (annealing), and 72°C for 1 minute (extension).

Plasmids VR1223 (luciferase) was constructed by cloning cytoplasmic luciferase gene into the VR1012 vector (Hartikka et al., *Hum. Gene Ther.* 7:1205-1217, 1996). The source of the cytoplasmic luciferase gene found in VR1223 was the plasmid termed pSP-luc+ which was purchased from Promega. An Avr II - Xba I restriction fragment encoding the luciferase cDNA was transferred from pSP-luc+ to VR1012 to make VR1223.

Plasmid VR1412 (β -galactosidase) was constructed by cloning cytoplasmic β -gal gene into the VR1012 vector (Doh et al., *Gene Ther.* 4:648-663).

Plasmid VR1332 was constructed by inserting a *SalI*-*Bam*HI fragment encoding chloramphenicol acetyltransferase (CAT) from pBS-CAT (Promega) into *SalI*/*Bam*HI-cut VR1012 vector (Hartikka et al., *Hum. Gene Ther.* 7:1205-1217 (1996)).

Example 2

Purification of pDNA

5 pDNA was transformed into *Escherichia coli* DH10B-competent cells
and grown in Terrific Broth (Sambrook, J. *et al.*, in: *Molecular Cloning: A*
laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring
Harbor, NY, p. A.2 (1989)) complemented with 50 µg/ml kanamycin in a 1
Liter shaker flask. Cells were harvested by centrifugation at the end of the
10 exponential growth phase (approximately 16 hr), typically yielding 10 grams
of biomass net weight per liter. Covalently closed circular pDNA was isolated
by a modified lysis procedure (Horn, N.A. *et al.*, *Human Gene Therapy* 6:
565-573 (1995)) followed by standard double CsCl-ethidium bromide gradient
ultracentrifugation with an average yield of approximately 5 mg per liter.
15 Plasmids were ethanol precipitated and resolubilized in saline at 4°C and
dialyzed against saline. Endotoxin content was determined by the *Limulus*
Amebocyte Lysate assay (Associates of Cape Cod, Inc., Falmouth, MA). All
plasmid preparations were free of detectable RNA. Endotoxin levels were less
than 0.6 Endotoxin Units/µg of plasmid DNA. The spectrophotometric
20 A260/A280 ratios were between 1.75 and 2.0.

Example 3

In Vitro Evaluation of Biological Activity of IFN ω and IFN α

25 To assure that the interferon plasmid DNA used in the following
examples encoded biologically active interferon, cell proliferation and
antiviral assays were performed. All culture medium used in this and
30 following examples was obtained from Life Technologies (Gaithersburg,
MD), and all serum was obtained from HyClone (Logan, Utah).

 UM449 cells (American Type Culture Collection, Rockville, MD)
were plated at a concentration of 2×10^5 cells per well in a 6 well plate and
incubated for 24 hours. Plasmid DNA and the lipid, DMRIE/DOPE (1:1) were

each diluted to a concentration of 1 mg in 0.5 ml Optimem medium (Life Technologies, Gaithersburg, MD). The lipid DMRIE/DOPE consists of the cationic lipid (+)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (DMRIE) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE) at a 1:1 mol:mol ratio (Felgner et al., *J. Biol. Chem.* 269:2550-2561, 1994). DMRIE/DOPE has been shown to be effective for both *in vitro* (Felgner et al., *J. Biol. Chem.* 269:2550-2561, 1994) and *in vivo* transfection (Stopeck et al., *J. Clin. Oncol.* 15:341-349, 1997 and Rubin et al., *Gene Ther.* 4:419-425, 1997). The lipid mixture and the DNA mixture were then gently mixed. Medium was removed from the cells which were rinsed gently with PBS, followed by addition of the DNA:lipid mixture (1 ml/well). After incubating the cells for 4-5 h at 37°C, one ml of Optimem with 30% fetal calf serum (FCS) was added to each well. Following an overnight incubation at 37°C, one ml of Optimem with 10% FCS was added to each well. Tissue culture supernatants were collected 48 h after the start of the *in vitro* transfection.

a. Antiproliferative activity

To evaluate the antiproliferative and hence, anti-tumor activity of IFN ω and IFN α , supernatants from the above described UM449 cells transfected with the interferon or control plasmid DNA were tested in a cell proliferation assay of murine or human tumor cell lines (ATCC, Rockville, MD) using the Boehringer Mannheim (Indianapolis, IN) Cell Proliferation Kit II (XTT). Murine or human tumor cells were plated in 96 well plates at the desired concentration (cell concentration varied with each cell line evaluated, for example, at a concentration of 5×10^3 cells/ml for B16F10 cells and 5×10^4 cells/ml for the Cloudman S91 and glioma 261 cells). The plates were incubated at 37°C for 24 hours followed by addition of tissue culture supernatants from UM449 cells *in vitro* transfected with either interferon plasmid DNA or control plasmid DNA. As a positive control for mIFN α plasmid DNA, mIFN α protein (ICN Pharmaceuticals

Inc., Costa Mesa, CA) was serially diluted and added to the wells. For the hIFN plasmid DNA, an interferon reference standard (human leukocyte interferon, Sigma Chemical Co., St. Louis, MO) was included in each assay. Following a 24-72 hour incubation, at 37°C, 50 µl of XTT/ECR substrate was added to each well. Plates were incubated for 6-24 hours at 37°C and the optical density (OD) at 490 nm was determined. Increasing amounts of interferon result in inhibition of cell proliferation and a reduction in the OD₄₉₀. The percent reduction in cell proliferation due to addition of the supernatants was determined by the formula:

$$1 - \frac{\text{OD}_{490} \text{ of cells incubated with interferon plasmid DNA supernatants}}{\text{OD}_{490} \text{ of cells incubated with control plasmid DNA supernatants}} \times 100$$

As shown in Table 1, both human interferons displayed the characteristic potent anti-proliferative activity against a wide variety of human tumor cell lines, with the most sensitive line being the NIH-OVCAR3 ovarian line and the least sensitive being the SK-OV-3 ovarian line. Also, the supernatants from the mIFN α pDNA (VR4111)-transfected UM449 cells inhibited the proliferation of murine B16F10 melanoma (generous gift from Dr. Suzuki at the University of Texas, Galveston, Texas), murine Cloudman melanoma S91 (American Type Culture Collection, Rockville, MD), and murine glioma 261 cell lines (Division of Cancer Treatment Tumor Repository, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD) by 40, 42 and 17%, respectively.

Table 1. IFN ω (VR4150) and IFN α (VR4102) *in vitro* biological assay: anti-proliferation activity against human tumor cell lines

Cell line (tissue type)	% reduction in cell proliferation (compared to control plasmid DNA supernatants)	
	Interferon ω	Interferon α
NIH-OVCAR3 (ovarian)	60	43
SCC-4 (squamous)	36	36
ACHN (renal)	41	35
A431 (epidermoid)	24	19
SCC-15 (squamous)	29	29
U87MG (glioblastoma)	36	30
A375 (melanoma)	24	21
PC3 (prostate)	20	22
UMUC3 (bladder)	14	6
A549 (lung)	17	15
MCF7 (breast)	18	18
SK-OV-3 (ovarian)	< 10	< 10

b. Antiviral Activity

An antiviral assay was performed to evaluate the ability of the supernatants from the interferon plasmid DNA-transfected cells to protect murine L929 cells or human A549 cells from infection by murine encephalomyocarditis (EMC) virus (Assay performed at IIT Institute, Chicago, IL). *In vitro* transfections were performed as described above and supernatants were collected from cells transfected with either VR4151 (hIFN ω), VR4112 (hIFN α), VR4111 (mIFN α) or VR1055 (control). Antiviral activity of the supernatants was performed by IIT Research Institute (Chicago, IL). The antiviral assay evaluated the degree of protection of either human A549 or murine L929 cells from infection with murine encephalomyocarditis (EMC) virus. Briefly, 2.5×10^4 L929 cells were plated into 96-well plates and incubated for 24 h. Tissue culture supernatants were serially diluted and added to the L929 cells which were

incubated for another 24 h. Supernatants were then removed from the wells, the cells were washed and murine EMC virus was added to each well at a multiplicity of infection of 0.04. Assay plates were incubated further for 24 h followed by removal of supernatants, washing of wells, fixation with 5% formalin and staining with 1% crystal violet. Samples with interferon activity protected the cells from virus infection, resulting in darkly stained cell monolayers.

Supernatants from UM449 cells transfected with VR4151, VR4112, or VR4111 had antiviral activity of 30,000, 3,000 or 30 Units/ml, respectively, on human A549 cells. When evaluated for antiviral activity on the murine L929 cell line, supernatants from UM449 cells transfected with VR4151, VR4112, or VR4111 had antiviral activity of 300, 1000 and 30,000 Units/ml, respectively (Table 2) showing species specificity of the hIFNs for human cells and mIFNs for mouse cells.

Table 2. Antiviral Activity of interferon Plasmid DNA

Plasmid	Interferon (Units/ml)	
	Human cell line	Murine cell line
VR4151	30,000	300
VR4112	3,000	1,000
VR4111	30	30,000

Example 4

Systemic Interferon Therapy; Intramuscular Administration of Cytokine-Expressing Plasmids

Cell lines and tumor models

Murine B16F10 cells were grown in RPMI-1640 (GibcoBRL) and 5% fetal bovine serum (FBS). Murine Cloudman S91 cells were grown in Ham's F-10 medium with 25 mM Hepes, 0.1 mM, non-essential amino acids, 1 mM sodium pyruvate, 0.05 mM β -mercaptoethanol, 2.5% FBS and 12.5% horse serum. Human melanoma UM449 cells were grown in RPMI 1640 with 10% FBS.

Murine glioma 261 tumor fragments and M5076 reticulum cell sarcoma cells were obtained from the Division of Cancer Treatment Tumor Repository (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD). The glioma 261 tumor fragments (2mm³) were initially implanted into the inguinal region of C57BL/6 mice using a 13 g trocar (Popper Sons, Inc., New Hyde Park, NY). Tumors which grew in the mice were used to establish a tumorigenic cell line. Minced tumor fragments were placed in Iscove's tissue culture medium with 10% FBS. Glioma 261 tumor cells began to attach to the flasks after several days, and the cells were propagated using standard tissue culture techniques. The M5076 cells were grown as ascites in C57BL/6 mice and frozen in liquid nitrogen. Human A431 cells were obtained from the American Type Culture Collection and were grown in DMEM and 10% FBS.

C57BL/6, DBA/2, nude (*nu/nu*) and beige-nude (*bg/nu/xid*) female mice between the ages of 6-8 weeks were obtained from Harlan Sprague Dawley (San Diego, CA). All animal experiments in this and the following examples were conducted in accordance with Vical's Institutional Animal Care and Use Committee as well as the standards set forth in the National Research Council guidelines concerning animal care and use.

To establish subcutaneous B16F10 melanoma tumors, C57BL/6, nude or nude-beige mice were injected subcutaneously on the flank with 10⁴

B16F10 cells. The Cloudman melanoma model was established by subcutaneous injection of 10^5 Cloudman S91 cells on the flank of DBA/2 mice and the glioma 261 model was established by subcutaneous injection of 5×10^4 glioma 261 cells on the flank of C57BL/6 mice. To establish human epidermoid carcinomas, nude mice were injected subcutaneously on the flank with 5×10^3 A431 cells.

To establish intradermal M5076 tumors and liver metastases thereof, C57BL/6 mice were injected intradermally with 10^5 M5076 reticulum cell sarcoma cells. In this model, primary intradermal tumors spontaneously metastasize to the liver. On day 29 after tumor cell injection, mice were sacrificed, livers removed and fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA), and the liver nodules were counted.

To establish lung metastases of B16F10 melanoma, C57BL/6 mice were injected intravenously with 2×10^4 B16F10 cells. On day 25 after tumor cell injection, the mice were sacrificed, lungs removed and fixed in 10% buffered formalin, and the lung nodules were counted.

To monitor the primary tumor growth, tumor sizes were determined 2-3 times per week by measuring with calipers ($1 \times w \times h$), and tumor volumes were determined using the formula: tumor volume (mm^3) = $0.52 (1 \times w \times h)$.

Tumor volume was analyzed using the Mann-Whitney U non-parametric Statistical Test to identify groups having significantly different mean weights. Mouse survival was analyzed using a Kaplan-Meier survival plot followed by a Logrank (Mantel-Cox) test to identify significant differences in survival between groups. Differences were considered statistically significant when the p value was less than or equal to 0.05.

Intramuscular injections

Fifty to 100 μg of plasmid DNA in 50 μl of saline was injected into the rectus femoris muscle of each hind leg for a total DNA dose of 100 to 200 μg . The muscle injections were performed using a 300 μl sterile tuberculin syringe fitted with a 28G $\frac{1}{2}$ needle (Becton Dickenson) and a plastic collar cut from a

200 µl micropipette tip. The collar length was adjusted to limit the needle from penetrating further than 2 mm into the rectus femoris muscle.

5 *Serum levels of interferon following intramuscular injection of
interferon plasmid DNA*

10 Serum samples from C57BL/6 mice injected intramuscularly with
either VR4111 (mIFN α plasmid) or VR1055 (control plasmid DNA) were
analyzed in a mIFN α ELISA (n=10). For the ELISA, 96-well plates
(Immulon 4HBX high binding plates from Dynex Technologies, Chantilly,
VA) were coated with rat anti-mouse IFN α monoclonal antibody (mAb) from
Caltag Laboratories (Burlingame, CA) at a concentration of 5 µg/ml in 100
mM sodium carbonate buffer, pH 9.5 (50 µl per well). Plates were incubated
with the coating mAb for 16 hours at 4°C. The plates were then washed 3
15 times with a wash buffer (phosphate-buffered saline (PBS)), pH 7.2 and 0.05%
Tween-20 (Sigma, St. Louis, MO)). The plates were blocked in PBS
containing 3% bovine serum albumin (BSA, Sigma) and 0.05% Tween-20
(400µl per well) and incubated for 24 hours at 4°C followed by washing three
times with wash buffer.

20 Serum samples (10 µl) from mice injected intramuscularly with
VR4111 (mIFN α) were mixed with 40 µl of assay buffer (PBS, 1% BSA,
0.05% Tween-20) and the mixture was added to each assay well. The positive
control was mIFN α polypeptide (Biosource International, Camarillo, CA),
which was serially diluted in assay buffer and 50 µl was added to the positive
25 control wells. The negative control was serum from mice injected
intramuscularly with VR1055. After adding the test samples and controls, the
plates were incubated for 16 hours at 4°C. The plates were then washed 6
times with wash buffer, followed by addition of a sheep anti-mouse IFN α
polyclonal antibody (pAb) (Biosource International, Camarillo, CA). The
30 PAb was added at a 1:500 dilution in assay buffer (50 µl per well) and the
plates were incubated for 5 hours at room temperature.

Following incubation with the pAb, the plates were washed six times with wash buffer followed by addition of anti-sheep IgG conjugated with peroxidase (Sigma) at a 1:5000 dilution in assay buffer (50 μ l per well) and incubated for 1 hour at room temperature. The plates were washed 6 times with wash buffer and 200 μ l of 3,3',5,5'-tetramethylbenzidine liquid substrate (TMB) (Sigma Chemical Co.) was added per well. Plates were incubated at room temperature for 30 minutes, followed by determination of the optical density of the wells at 650 nm. A standard curve was generated by plotting the ng/ml of mIFN α polypeptide versus the optical density at 650 nm. The concentration of mIFN α in the test serum samples was determined from the linear portion of the mIFN α standard curve. The sensitivity of the mIFN α ELISA was 50 ng/ml.

C57BL/6 mice injected intramuscularly with VR4111 had detectable serum levels of mIFN α after 5 intramuscular injections of 100 μ g VR4111 (twice a week for two weeks, followed by one injection the next week). The average serum level of mIFN α after 5 intramuscular injections of VR4111 was 1465 ng/ml (average of 16 mice). At the time of this study, no commercial mIFN α ELISA kit had been developed. Since sensitivity of the in-house mIFN α ELISA is 50 ng/ml, lower serum levels of mIFN α could exist in the mice at earlier timepoints, but we were unable to detect this in our ELISA.

To determine the serum levels of hIFN ω , C57BL/6 or nude mice received a single intramuscular injection of 100 μ g of VR4151 (hIFN ω plasmid DNA) or VR1055 (control plasmid DNA) (50 μ g per leg bilaterally) in the rectus femoris. Serum samples were collected daily over a two week period and analyzed in the hIFN ω ELISA kit (Alexis, San Diego, CA) which was sensitive to 2 pg/ml. Serum samples were collected from 4-5 mice per day. In the C57BL/6 mice, measurable serum levels of hIFN ω were detected as early as one day after injection (69 pg/ml) (Figure 2A). In these mice, peak serum levels were found six days after injection (254 pg/ml) and expression continued out to day 14 (50 pg/ml), the final timepoint of the study.

In nude mice, serum levels of IFN ω were found as early as one day after injection (133 pg/ml). Peak serum levels were found on day 7 (648 pg/ml) and expression continued out to day 14 (134 pg/ml), the final time point of the study (FIG. 2B). Thus, interferon could be detected in the serum after a single intramuscular injection of an interferon-encoding plasmid DNA.

Systemic interferon treatment inhibits primary tumor growth

As shown in FIGs. 3-5 and FIG. 7A, mice bearing different tumors were found to significantly benefit from intramuscular injection of different cytokines. To test the efficacy of IFN α plasmid, C57BL/6 mice bearing subcutaneous B16F10 melanoma, subcutaneous glioma 261, or intradermal M5076 tumors, or DBA/2 mice bearing subcutaneous Cloudman melanoma were injected with 100 μ g either of VR4111 (mIFN α) or VR1055 (control), twice per week for three weeks, beginning on day 4 after tumor cell injection (n = 8-10 mice per group). In all three subcutaneous tumor models, the mice treated intramuscularly with VR4111 had a significant reduction in tumor volume (p<0.05) (FIGs. 3A, 3C, and 3E), and a significant enhancement of survival (p<0.02) compared to the mice that received the control plasmid (FIGs. 3B, 3D, and 3F). In the intradermal tumor model, mice treated with intramuscular VR4111 had a significant reduction in primary tumor volume (p<0.001) compared to the mice that received the control plasmid (FIG. 7A).

To compare the efficacy of IL-2, IL-12 to IFN α plasmids, C57BL/6 mice bearing subcutaneous B16F10 melanoma were injected with 100 μ g of VR4111 (mIFN α), VR4001 (mIL-12), VR1110 (mIL-2), or VR1012 (control) (n = 15-16 mice per group) twice per week for three weeks. Mice receiving intramuscular injections of VR4111 had a significant reduction in tumor growth (p < 0.0002) (FIG. 4A) by day 17 as well as a significant increase in survival (p = 0.00001) (FIG. 4B). By day 28 of the study, 100% of the VR4111-treated mice were still alive, compared to only 20% of the VR1012-treated mice. Mice treated with VR1110 had a modest reduction in tumor

growth by day 17 ($p < 0.02$) (FIG. 4A) but did not have an increase in survival compared to the VR1012-treated mice (FIG. 4B). Mice treated with VR4001 also had a modest reduction in tumor growth by day 17 ($p < 0.03$) (FIG. 4A) as well as a significant increase in survival ($p = 0.02$) (FIG. 4B). By day 28, 55% of the mice treated with VR4001 were alive, compared to 20% of the VR1012-treated mice.

To test the efficacy of IFN ω , mice bearing human A431 tumors between 30-80 mm³ were injected intramuscularly with 200 μ g of either VR4151 (hIFN ω) or VR1055 (control) twice per week for three weeks ($n=15$). Mice bearing subcutaneous A431 tumors and injected intramuscularly with VR4151 had a significant reduction in tumor volume ($p < 0.05$) (FIG. 5A) and a significant increase in survival ($p < 0.05$), compared to the mice that received the control plasmid (FIG. 5B).

Systemic mIFN α plasmid DNA treatment inhibits the growth of tumor metastases

As shown in FIG. 6 and FIG. 7, mice bearing different tumor metastases were found to significantly benefit from intramuscular injection of IFN α . C57BL/6 mice bearing lung metastases of B16F10 melanoma were injected intramuscularly with 100 μ g of either VR4111 (mIFN α) or VR1055 (control) twice per week for three weeks, beginning on day 4 after tumor cell injection ($n=10$). On day 25 after tumor cell injection, the mice were sacrificed, lungs removed and fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA) followed by counting of lung nodules.

While 70% of the control plasmid-treated mice had lung nodules that were too numerous to count, 80% of the mice treated with mIFN α plasmid DNA had 10 or fewer nodules (FIG. 6). TNTC denotes lungs with nodules that were too numerous to count.

In the liver metastases model, C57BL/6 mice bearing intradermal M5076 murine reticulum sarcoma were injected intramuscularly with 100 μ g of either VR4111 or VR1055 twice per week for three weeks, beginning on

day 4 after tumor cell injection (n=10-13 mice per group). On day 29 after tumor cell injection, the mice were sacrificed, livers removed and fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA) followed by counting of liver nodules.

5 While the control plasmid-treated mice had a mean of 190 hepatic tumor nodules or had nodules that were too numerous to count, mIFN α plasmid DNA-treated mice had a mean of 35 hepatic tumor nodules (FIG. 7B).

10 These results demonstrate that intramuscular injection of mIFN α plasmid DNA can effectively inhibit the growth of both primary and metastatic lesions. Thus, for patients with metastatic disease, intramuscular administration of therapeutic plasmid DNAs would be advantageous for the treatment of undiagnosed or inaccessible metastatic lesions.

15 *Regimen optimization of mIFN α therapy in the B16F10 melanoma model*

20 A regimen optimization study was conducted to evaluate the antitumor efficacy of fewer injections and/or a lower dose of VR4111(mIFN α) in the subcutaneous B16F10 melanoma model. C57BL/6 mice were injected with either 100 or 50 μ g of VR4111 or VR1055 over a 6 week period (n=10). Mice received intramuscular injections either twice per week, once per week or once every other week. All intramuscular injections began four days after the initial subcutaneous B16F10 tumor cell injection. Mice which received intramuscular injections of 100 μ g of VR4111 at any of the time courses had a
25 significant reduction in tumor volume ($p \leq 0.005$) and a significant increase in survival ($p = 0.007$) (FIGs. 8A and 8B). The mice which received the 100 μ g dose of VR4111 once every other week for 6 weeks had a total of only three intramuscular injections with significant antitumor efficacy. In contrast, mice receiving the 50 μ g dose of VR4111 revealed a dose response based on the
30 frequency of injection. While mice injected with 50 μ g of VR4111 once or twice per week had a significant reduction in tumor volume ($p \leq 0.03$), and a

significant increase in survival ($p=0.002$), mice injected only once every other week did not have a significant antitumor response for either tumor growth or survival (FIGs. 8C and 8D).

Mechanism of mIFN α Antitumor Effect

To investigate the role of natural killer (NK) and T cells in mediating the antitumor effect of systemically delivered mIFN α , VR4111 or VR1055 was administered intramuscularly to nude mice (which are T cell deficient) and to beige-nude mice (which are NK and T cell deficient) bearing subcutaneous B16F10 melanoma tumors. Beginning on day 4 after injection of 10^4 B16F10 cells, 50 μ g of plasmid DNA in 50 μ l of saline was injected into the rectus femoris muscle of each hind leg for a total DNA dose of 100 μ g twice per week for three weeks ($n=15$ mice per group).

There was neither a significant reduction in tumor volume nor enhancement of survival in the nude mice (FIGs. 9A and 9B) or beige-nude mice (FIGs. 9C and 9D). These results suggest that T cells may be required for the mIFN α antitumor response. NK cells appeared not to be required for the antitumor effect since nude mice (NK $^+$) did not have a greater antitumor response compared to the beige-nude mice (NK $^-$).

To further explore the role of T cells in the antitumor effect of mIFN α DNA therapy, C57BL/6 mice bearing subcutaneous B16F10 tumors were injected with depleting doses of monoclonal antibodies (mAbs) specific for either CD4 $^+$ or CD8 $^+$ T cells. For depletion of T cell subsets, anti-CD4 (clone GK1.5, rat IgG) and anti-CD8 (clone 2.43, rat IgG) hybridomas (American Type Culture Collection, Rockville, MD) were used to generate the corresponding mAb. The anti-CD8 hybridoma was grown as ascites in nude mice and the mAbs were purified from ascites using ion exchange chromatography (Harlan Bioproducts for Science, San Diego, CA). The anti-CD4 hybridoma was grown *in vitro* with Dulbecco's Modified Eagle Medium, 10% fetal bovine serum and low IgG. The anti-CD4 mAb was purified from

tissue culture supernatant by ammonium sulfate precipitation to 30%. The protein pellet was resolubilized and extensively dialyzed in Dulbecco's $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (Zymed Laboratories Inc., San Francisco, CA).

5 Beginning on day 4 after subcutaneous injection with 10^4 B16F10 cells, mice were injected intramuscularly with 100 μg of either VR4111 or VR1055 twice per week for three weeks. For depletion of CD4^+ and CD8^+ T cells, mice were injected intraperitoneally with 500 μg of either the anti-CD4 mAb (clone GK1.5, rat IgG) or anti-CD8 mAb (clone 2,43, rat IgG) one day
10 prior to each intramuscular DNA injection ($n = 10$ mice per group). Control tumor-bearing mice were injected intraperitoneally with 500 μg of normal rat IgG (Simga Chemical Co., St. Louis, MO) ($n=10$). To assure complete depletion, sentinel mice were injected according to the same regimen, and once per week, spleens were collected, dissociated and assessed for the
15 presence of CD4^+ and CD8^+ T cells. Spleen cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 mAbs (Pharmingen, San Diego, CA) and analyzed by flow cytometry (Cytometry Research Services, San Diego, CA). The depletion of CD4^+ and CD8^+ T cells was consistently greater than 98%, as determined by cytometry.

20 The mIFN α DNA therapy significantly reduced tumor growth ($p \leq 0.002$) and enhanced survival ($p \leq 0.008$) of both normal mice and mice depleted of CD4^+ T cells, compared to mice injected with control plasmid and treated with normal IgG (FIGs. 10A and 10B). These results suggest that
25 CD4^+ T cells are not required for the mIFN α antitumor effect. In contrast, mice depleted of CD8^+ T cells and injected with mIFN α DNA displayed tumor volumes and survival profiles that were not significantly different from mice treated with the control plasmid (FIGs. 10A and 10B). This result suggests that CD8^+ cells are involved in the mIFN α antitumor response.

Example 5

Local Interferon Therapy: Intratumoral Administration of interferon Plasmids

5 The anti-tumor activity of IFN ω and IFN α was evaluated *in vivo* in nude mice bearing subcutaneous human ovarian (NIH-OVCAR3) or human melanoma (A375) (nude/human/xenograft model), or in C57BL/6 mice bearing murine melanoma (B16F10) tumors following intratumoral
10 administration of DNA complexed with a cationic lipid.

Cell Lines and Tumor Models

15 Athymic nude (*nu/nu*) and C57/BL/6 mice between the ages of 6-10 weeks were obtained from Harlan Sprague Dawley (San Diego, CA).

Human A375 melanoma cells and human NIH-OVCAR3 ovarian carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD), and grown in Dulbecco's modified Eagle's medium (GibcoBRL, Gaithersburg, MD) supplemented with 10% FBS. B16F10 cells
20 were a generous gift from Dr. Suzuki at the University of Texas (Galveston, Texas). Cells were grown in RPMI-1640 (GibcoBRL) and 5% fetal bovine serum (FBS).

To establish subcutaneous A375 melanoma tumors and subcutaneous NIH-OVCAR3 ovarian tumors, athymic nude/nude mice (10 mice/group)
25 were injected subcutaneously with 5×10^6 A375 cells and 5×10^7 NIH-OVCAR3 cells respectively. To establish subcutaneous murine B16F10 melanoma tumors, C57BL/6 mice were injected subcutaneously with 10^4 B16F10 cells.

30 Mice were monitored for tumor growth and survival. Tumor sizes were determined 3 times per week by measuring with calipers (l x w x h) and tumor volumes were determined using the formula: tumor volume (mm³) = $0.52 (l \times w \times h)$. Statistical analysis was done as described in Example 4.

Local Interferon Plasmid DNA Inhibits Tumor Growth

Established subcutaneous A375 human melanoma and NIH-OVCAR3 human ovarian carcinoma tumors in nude mice were transfected *in vivo* by intratumoral administration of pDNA/DMRIE/DOPE (DNA: lipid) complexes (n=10). When tumors became palpable (80-300 mm³, at day 27 post tumor cell implant for the A375 cells, and at day 41 post tumor cell implant for the NIH-OVCAR3 cells), mice were injected intratumorally with 100 µg of VR4112 (hIFNα), VR4151 (hIFNω), VR1055 (control) or VR1012 (control) complexed with DMRIE/DOPE (1:1 DNA:DMRIE mass ratio). Tumor bearing animals were treated intratumorally with DNA: lipid for 6 consecutive days followed by 5 treatments every other day for a total of 11 treatments (A375 melanoma model), or every other day for a total of 11 treatments (NIH-OVCAR3 ovarian cancer model).

As shown in FIG. 11A, in the A375 model of melanoma, the direct intratumoral injection of VR4151: lipid complex for 6 consecutive days, followed by 5 additional injections every other day (100 µg plasmid DNA/injection, total of 11 injections), resulted in a statistically significant slowing of tumor growth, as compared to the control (p < 0.03, days 40-44).

As shown in FIG. 2B, in the NIH-OVCAR3 model of ovarian cancer, the direct intratumoral injection of VR4151: lipid complex every other day for a total of 11 injections (100 µg plasmid DNA/injection), resulted in sustained and statistically significant reduction in tumor growth as compared to the control plasmid control (p < 0.001 - 0.05, Days 45-65). A similar treatment regimen with VR4112: lipid was found to have a moderate effect on tumor growth which only reached statistical significance vs. the control plasmid at two time-points during the study (p < 0.05, days 53 and 57).

As shown in FIG. 12A, in the B16F10 melanoma model, direct intratumoral injection of mIFNα resulted in decrease in tumor volume. Once palpable tumors were established (80-300mm³) on day 12 after tumor cell injection in C57BL/6 mice bearing subcutaneous B16F10 melanoma tumors,

mice were injected intratumorally with 100 µg of either VR4101 (mIFNα) or VR1012 (control) complexed with the cationic lipid, DMRIE/DOPE at a DNA/DMRIE mass ratio of 1:1 in a 100 µl volume. Mice received six consecutive intratumoral injections of either VR4101 or VR1012 (n=10 mice per group). As shown in FIG. 12A, although not statistically significant, intratumoral injection of VR4101 resulted in a 54% reduction in tumor volume by day 19 of the study. As shown in FIG. 12B, a significant increase in survival (p = 0.02) was found for the VR4101-treated mice, compared to the mice that received VR1012.

Example 6

Local Cytokine Therapy: Intraperitoneal Administration of Cytokine-expressing Plasmids

The goal of this example is to show that the present invention provides an effective method of treating malignant tumors of murine ovarian carcinoma via intraperitoneal (i.p.) injection of cytokine-expressing plasmid DNA. Since late-stage ovarian carcinoma is usually limited to the peritoneal cavity, it was envisioned that continuous secretion of a cytokine in this cavity would produce beneficial anti-tumor immune response. In particular, the present example clearly shows that the ovarian cancer therapy by intraperitoneal injection of a cytokine-expressing plasmid DNA:lipid complexes (1) results in sustained levels of the cytokine in the ascites, avoiding the need for frequent injections of the protein (in contrast to intraperitoneal injection of recombinant cytokine wherein the cytokine level declines shortly after injection), (2) targets tumor ascites, rather than peritoneal tissues (suggesting that systemic cytokine side effects should be reduced using this method), (3) inhibits tumor growth and enhances survival, and (4) can be combined with debulking of tumor ascites to enhance the antitumor effect.

Cell Lines and Tumor Models

As a model for human ovarian cancer, the murine ovarian teratocarcinoma (MOT) model in C3H/HeN mice was used. MOT exhibits many of the characteristics of late-stage human ovarian cancer including peritoneal spread, production of tumor ascites and tumor cell blockage of lymphatics (Ozols et al., 1979 and Fekete et al., 1952).

Murine ovarian teratocarcinoma (MOT) cells were obtained from Dr. Robert Knapp and Dr. Robert C. Bast at the Dana-Farber Cancer Center (Boston, MA). The MOT cells (10^5) were grown by serial intraperitoneal (i.p) transplantation in C3H/HeN mice and a stock of the cells was frozen in liquid nitrogen.

CTLL-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were grown in RPMI 1640 with glutamine, 1% sodium pyruvate, 1% penicillin-streptomycin (Life Technologies, Gaithersburg, MD), 10% fetal bovine serum (HyClone, Logan, Utah) and 10 U/ml murine IL-2 (Boehringer Mannheim, Indianapolis, IN).

C3H/HeN and nude (*nu/nu*) female mice between the ages of 6-10 weeks were obtained from Harlan Sprague Dawley (San Diego, CA). All animal experiments were conducted in accordance with Vical's Institutional Animal Care and Use Committee as well as the standards set forth in the National Research Council guidelines concerning animal care and use.

To establish i.p. MOT tumors, C3H/HeN mice were injected i.p. with 10^5 MOT cells in 100 μ l of medium. In the MOT tumor model, tumor growth is typically monitored by weighing the mice which reflects the increase in volume of tumor ascites (Berek et al., *Cancer Res.*, 44:1871-1875, 1984). The nude mouse study were performed in the same manner as the studies in C3H/HeN mice with injection i.p. of 10^5 MOT cells and monitoring the weight of the mice. Statistical analysis on mouse weight and survival was done as described in Example 4.

Preparation of plasmid DNA:lipid complexes and intraperitoneal injection

To yield a pDNA:DMRIE mass ratio of 1:1, 100 µg of VR1110 (mIL-2) was diluted in 500 µl 0.9% saline (Radix Labs, Eau Claire, WI), DMRIE/DOPE lipid (100 µg DMRIE) was diluted in 500 µl of 0.9% saline in a separate vial, and the pDNA and cationic lipid were combined and vortexed for 5 seconds. To yield a pDNA:DMRIE mass ratio of 5:1, 500 µg of mIL-2 pDNA was diluted in 500 µl 0.9% saline (Radix Labs, Eau Claire, WI), DMRIE/DOPE lipid (100 µg DMRIE) was diluted in 500 µl of 0.9% saline in a separate vial, and the pDNA and cationic lipid were combined and vortexed for 5 seconds.

The 1 ml pDNA:DMRIE/DOPE (DNA: lipid) complex was injected i.p. into mice bearing i.p. MOT tumors on various days after tumor cell implant. Control MOT tumor-bearing mice received i.p. injections of VR1012 (control): lipid at the same ratio (1:1 pDNA:DMRIE, 100 µg pDNA) and were injected i.p. on the same days as the cytokine-expressing or reporter gene treatment groups.

Intraperitoneal injection of plasmid DNA:lipid results in targeted expression in tumor ascites

The pDNA:lipid therapy was evaluated for the ability to target malignant cells within a cavity. C3H/HeN mice were injected i.p. with 10^5 MOT cells followed by i.p. injection of 100 µg of VR1223 (luciferase): lipid (1:1 pDNA:DMRIE mass ratio) on days 5 and 6 after tumor cell implant. Control MOT tumor-bearing mice were injected i.p. with 100 µg of either VR1012: lipid or VR1223 without lipid on days 5 and 6 after MOT tumor cell injection. An additional group of control mice did not receive MOT tumor cells and were injected i.p. with VR1223: lipid on the same days as the other treatment groups (n=3). Three days later the mice were euthanized and tumor ascites and tissues (liver, kidney, spleen, diaphragm, intestine and ovary) were

collected. Luciferase was extracted from the tissues by freeze-thawing and grinding of the samples in cell lysis reagent (Promega, Madison, WI) as previously described (Hartikka et al., *Hum. Gene Ther.*, 7:1205-1217, 1996). The tumor ascites was diluted 1:5 in cell lysis reagent followed by three cycles
5 of freeze-thaw and collection of supernatant from the cell lysate. Samples were read in a microplate luminometer (Dynatech, Chantilly, VA) following addition of luciferase substrate (Promega, Madison, WI). The relative light units (RLU) of the samples were determined from a standard curve using purified firefly luciferase (Analytical Luminescence Laboratory, Sparks, MD).
10 The protein concentration of each sample was determined using the BCA protein assay kit (Pierce Chemical Company, Rockford, IL). Luciferase levels were expressed as RLU per mg of protein. There may be a decrease in IL-2 pDNA expression from day 1 to day 3 post DNA injection.

On day three, tumor ascites had 900,000 RLU of luciferase/mg, while
15 diaphragm and ovary tissue had only 327 and 16 RLU/mg (FIG. 13). Kidney, liver, spleen and intestinal tissue had no detectable luciferase activity. These results suggest that i.p. injection of pDNA: lipid complexes appears to target the tumor ascites in the peritoneal cavity with limited or negligible transfection of surrounding tissues. Luciferase detected in the diaphragm and
20 ovary tissue was only found in MOT tumor-bearing mice injected with VR1223: lipid. When naive non-tumor bearing mice were injected with the same DNA: lipid complex, no luciferase activity was found in any of the tissues (data not shown). These results suggest that the low levels of luciferase in the diaphragm and ovary in MOT tumor-bearing mice may reflect
25 metastases of tumor cells to these tissues. Tumor-bearing mice injected with luciferase pDNA without cationic lipid had no luciferase activity in either tumor ascites or surrounding tissues, indicating that lipid is required for optimal *in vivo* transfection of ovarian tumor ascites. Mice injected with VR1012: lipid had no detectable luciferase activity in either tumor ascites or
30 tissues.

A follow-up study investigated the specific cell type in the ovarian tumor ascites that was transfected after i.p. injection of a reporter gene

pDNA:DMRIE/DOPE complex. On days 5 and 6 after tumor cell implant (10^5 cells), C3H/HeN mice were injected i.p. with 100 μ g of VR1412 (β -galactosidase (β -gal)): lipid, VR1012: lipid (1:1 DNA:DMRIE mass ratio), or with VR1412 without cationic lipid (n=3 mice per group). One day later the mice were sacrificed and the tumor ascites was collected. The ascites was spun at 2500 rpm for 2 minutes to pellet the cells, and the supernatant was removed. The tumor cells were fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA), placed in a cryomold containing OCT embedding medium (VWR, S. Plainfield, NJ), frozen in isopentane and then stored at -70°C . The embedded and frozen samples were then cryostat sectioned (5 μ m), further fixed (0.5% glutaraldehyde in PBS), washed (PBS), stained with X-gal reagent (1 mg/ml X-gal diluted in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mM magnesium chloride), washed again (PBS), and counterstained with hematoxylin and eosin. (The samples were cryostat sectioned and stained by Pathology Associates (Frederick, MD).)

The ascites from mice treated with either VR1012 or VR1412 without lipid had no β -gal activity in the samples. In contrast, the tumor ascites from mice injected with VR1412: lipid had β -gal staining primarily in the tumor cells (data not shown). In a few slides, several macrophages and lymphocytes were also positive for β -gal while neutrophils were negative for β -gal.

Intraperitoneal injection of IL-2 pDNA:lipid results in sustained expression of IL-2 in tumor ascites

A time-course study was done to determine the length of time that IL-2 could be detected after multiple i.p. injections of IL-2 pDNA: lipid or a single i.p. injection of either IL-2 protein or IL-2 pDNA: lipid in mice bearing i.p. ovarian tumor ascites. Beginning on day 5 after tumor cell injection, mice were injected multiple times with VR1110 (mIL-2) : lipid or a single time with VR1110: lipid or IL-2 protein. Mice were sacrificed at various times and ascites and serum were analyzed for IL-2 levels. Ascites was collected from

the sacrificed mice, the samples were spun at 14,000 rpm for 2 minutes and the supernatant was harvested. Blood was collected from the mice on the same day as the ascites collection and the serum was separated from blood cells by allowing the blood to clot in serum separator tubes (Microtainer, Becton Dickinson, Franklin Lakes, NJ) followed by centrifugation at 14,000 rpm for 10 minutes and collection of the serum supernatant. IL-2 concentration (pg/ml) in the ascites and serum samples was determined using a murine IL-2 ELISA (R & D Systems, Minneapolis, MN). Since the volume of tumor ascites increases over time, the volume of ascites was also determined for each mouse. The total concentration of IL-2 in ascites was determined using the formula: IL-2 pg/ml x ml of ascites = pg IL-2/ total ascites. Serum IL-2 concentrations were reported as pg/ml.

IL-2 in serum and ascites after multiple injections of IL-2 pDNA: lipid.

Beginning on day 5 after tumor cell injection, mice were injected with VR1110: lipid for either 2, 4 or 6 consecutive days or with control VR1012: lipid for 6 consecutive days (100 µl of plasmid DNA complexed with 100 µl DMRIE/DOPE at 1:1 mass ratio in a total volume of 1 ml). An additional group of mice received VR1110 that was not complexed with DMRIE/DOPE. Every two days for up to 17 days after the DNA:lipid injections, 3-4 mice were sacrificed per treatment group and ascites and serum were collected and analyzed using mIL-2 ELISA assay as described above.

Two injections of VR1110: lipid (100 µl DNA per day, 1:1 DNA:cationic lipid mass ratio) into mice bearing i.p. MOT ovarian tumors yielded high levels of IL-2 protein in the tumor ascites. One day after VR1110: lipid i.p. injection, 28,000 pg/ml of IL-2 was measured in the tumor ascites (Table 3). IL-2 expression in the ascites continued for over two weeks after DNA:lipid injection with 750 pg/ml detected 17 days after the last pDNA:lipid injection. Mice injected with either four or six consecutive injections of VR1110: lipid also had high levels of IL-2 in the tumor ascites;

however, due to the very high expression levels after more frequent VR1110:
lipid injections, IL-2-mediated side effects were noted in the mice which did
not survive beyond day 9 or 13 after DNA injection. In contrast, two
consecutive injections of VR1110 plus DMRIE/DOPE did not cause
5 observable IL-2 side effects yet IL-2 expression levels remained high for over
2 weeks. The IL-2 expressed after i.p. DNA:lipid injection of MOT-bearing
mice appeared to remain localized in the peritoneal cavity as the IL-2 serum
levels after VR1110: lipid i.p. injection were always less than 10% of the
levels in the tumor ascites. Injection of VR1110 without lipid yielded very
10 low levels of IL-2 in ascites and serum (0-32 pg/ml). Injection of the control
vector, VR1012, resulted in only background levels of IL-2.

Table 3
mIL-2 concentration in ascites (pg/ml)

		Days post tumor cell injection								
	Treatment	<u>7</u>	<u>9</u>	<u>11</u>	<u>13</u>	<u>15</u>	<u>17</u>	<u>19</u>	<u>21</u>	<u>23</u>
5										
10	VR1110 (mIL-2) without DM/DP, 6 injs.	28	12	0	1	0	1	0		
	VR1012 (control) + DM/DP, 6 injs.	0	0	8	0	0	1	43		
15	VR1110 (mIL-2) + DM/DP, 6 injs.			7196	7716	7824	3895	3200		
	VR1110 (mIL-2) + DM/DP, 4 injs.		3524	4187	5968	3050	3984	2392	441	
20	VR1110 (mIL-2) + DM/DP, 2 injs.	28882	4750	11725	8047	1246	1445	1407	774	753
25										

mIL-2 concentration in serum (pg/ml)

		Days post tumor cell injection								
	Treatment	<u>7</u>	<u>9</u>	<u>11</u>	<u>13</u>	<u>15</u>	<u>17</u>	<u>19</u>	<u>21</u>	<u>23</u>
30										
35	VR1110 (mIL-2) without DM/DP, 6 injs.	0	4	0	32	0	0	0		
	VR1012 (control) + DM/DP, 6 injs.	0	0	0	0	0	0	62		
40	VR1110 (mIL-2) + DM/DP, 6 injs.			66	85	34	5	4		
	VR1110 (mIL-2) + DM/DP, 4 injs.		114	81	40	10	3	2	0	
45	VR1110 (mIL-2) + DM/DP, 2 injs.	625	418	181	76	6	0	1	0	0

IL-2 in serum and ascites after single injection of pDNA:lipid injection compared to protein injection.

Five days after i.p. injection of 10^5 MOT tumor cells, C3H/HeN mice
5 were injected with 100 μ g of either VR1110: lipid or VR1012: lipid (1:1
DNA:DMRIE mass ratio) or with 100 μ g of VR1110 without lipid. For the
IL-2 protein-treated group, mice were injected with 1 μ g recombinant murine
IL-2 protein (R & D Systems, Minneapolis, MN). The pDNA: lipid, pDNA
alone, or recombinant protein was injected i.p. in a total volume of 1 ml saline
10 per mouse. Five mice from each group were sacrificed beginning at 4 hours
and continuing on days 1, 2, 3, 6 and 10 post DNA or protein injection.
Ascites and serum were collected and analyzed using mIL-2 ELISA assay as
described above.

Mice injected i.p. with IL-2 protein had peak levels of IL-2 in ascites
15 (10 ng) at 4 hours after injection of IL-2 and a 1000-fold reduction in IL-2 one
day later (0.009 ng) (FIG. 14A). In contrast, mice injected with IL-2 pDNA:
lipid had peak IL-2 levels in ascites 2 days after injection (64 ng) and only a
2.6 fold reduction in IL-2 by 10 days after injection (25 ng) (FIG. 14A).
These results indicate that mice receiving IL-2 pDNA: lipid have more
20 sustained levels of IL-2 in the ascites compared to mice receiving IL-2 protein.
Tumor-bearing mice injected i.p. with either VR1012: lipid or VR1110
without cationic lipid had no IL-2 in the tumor ascites. In a related study,
MOT tumor-bearing mice injected i.p. with 10-fold less VR1110: lipid (10 μ g
DNA) still had detectable IL-2 in the tumor ascites 11 days after the VR1110:
25 lipid injection (data not shown).

Serum levels of IL-2 after either i.p. protein or DNA injection reflected
a similar pattern as that found in tumor ascites; however, the serum IL-2 levels
were markedly reduced compared to the ascites IL-2 levels. Four hours after
protein injection, IL-2 in the serum was 2.4 ng/ml and negligible by one day
30 after protein injection. Serum levels of IL-2 one day after VR1110: lipid
injection was 1 ng/ml and undetectable by 6 days after the injection (FIG.

14B). These results suggest that the majority of the IL-2 after either IL-2 protein or pDNA: lipid delivery remains in the peritoneal cavity.

5 ***Intraperitoneal injection of IL-2 plasmid DNA:lipid inhibits tumor growth and enhances survival***

10 *Six consecutive-day treatments.* The plasmid DNA:lipid therapy was evaluated for the ability to reduce tumor growth and to increase survival of mice with MOT tumors. On day 5 after MOT tumor cell injection, mice were injected i.p. with 100 µg of either VR1110 or VR1012, both complexed with DMRIE/DOPE. The plasmid DNA was complexed at either a 5:1 or 1:1 DNA:DMRIE mass ratio. An additional treatment group received VR1110 that was not complexed with lipid. A total volume of 1 ml DNA:lipid or DNA alone in physiological saline was injected i.p. The DNA treatments occurred over 6 consecutive days, beginning on day 5 (days 5-10). MOT tumor growth was measured by weighing the mice. All treatment groups consisted of 10 mice per group.

15 The high IL-2 expression level in ascites was accompanied by significant antitumor effects. Mice treated with VR1110: lipid on days 5-10 after tumor cell injection had a significant reduction in MOT tumor growth compared to the mice treated with the VR1012: lipid ($p=0.01$) (FIG. 15A). A significant enhancement in survival was also found for mice injected i.p. with VR1110: lipid ($p=0.05$) (FIG. 15B).

20 Complexing the pDNA with a cationic lipid seemed necessary for the antitumor effect as treatment of tumor-bearing mice with VR1110 without lipid was not effective (FIGs. 15E and 15F). Furthermore, a 1:1 DNA:cationic lipid mass ratio was found to be more effective at reducing tumor burden and increasing survival than a 5:1 DNA:cationic lipid mass ratio (FIGs. 15C and 15D).

25 *Three alternative-day treatments.* C3H/HeN mice bearing i.p. MOT tumor ascites were injected i.p. with VR1110: lipid or with VR1012: lipid (100 µg DNA) on days 5, 8 and 11 after tumor cell implant. By day 14 post

tumor cell injection, mice treated with VR1110: lipid had a significant reduction in mean weight ($p=0.001$) compared to the mice treated with the control pDNA: lipid (FIG. 16A). In addition, a significant increase in survival ($p=0.008$) was found for the VR1110: lipid-treated mice compared to the mice treated with the VR1012: lipid (FIG. 16B). By day 26 post tumor cell injection, none of the mice treated with the VR1012 were still alive, while 50% of the mice treated with VR1110: lipid remained alive. By day 55 post tumor cell injection, 20% of the mice treated with VR1110: lipid appeared to be tumor-free.

Whether the IL-2 pDNA: lipid antitumor effect required T cells was investigated by implanting nude mice with i.p. MOT tumors followed by same VR1110: lipid regimen used in the C3H/HeN tumor-bearing mice (DNA treatment on days 5, 8 and 11 after tumor cell implant). No significant antitumor effect was found for the nude mice treated with VR1110: lipid suggesting that T cells may be required for the antitumor effect (data not shown).

IL-2 plasmid DNA:lipid antitumor effect enhanced by debulking of tumor ascites

Debulking of tumor ascites is commonly performed on human ovarian cancer patients. A similar procedure was performed in the mice bearing MOT tumors and treated with VR1110: lipid. Mice bearing MOT tumors and injected i.p. with 100 μ l DNA:lipid on days 5-10 as described above (1:1 DNA:lipid mass ratio), were also debulked of tumor ascites on day 14 after tumor cell injection (4 days after the last DNA:lipid injection). Mice were debulked of 5 ml of tumor ascites by insertion of a 22 G needle attached to a 5 ml syringe and removal of 5 ml of fluid. The mice were anesthetized with methoxyflurane during the debulking procedure. All treatment groups in this experiment consisted of 8-10 mice per group.

Debulking of ovarian tumor ascites in mice previously treated with IL-2 plasmid DNA:lipid further enhanced the efficacy of the treatment resulting

in a significant reduction in tumor growth ($p=0.01$) and an increase in survival. Forty four percent of the IL-2 plasmid DNA:lipid-treated and debulked mice were alive on day 57 vs. 17% of the plasmid control-treated and debulked mice (FIG. 17). These results show that plasmid-mediated gene therapy in combination with conventional procedures such as debulking of tumor ascites may hold promise for future treatment of human ovarian cancer.

Dose-response of IL-2 pDNA: lipid

A dose-response study was initiated to determine the minimum dose of VR1110: lipid that could still result in a significant antitumor effect. C3H/HeN mice were injected with 25, 50 or 100 μ g of IL-2 pDNA: lipid on days 5, 8 and 11 after MOT tumor cell injection. A control group of MOT tumor-bearing mice were injected with 100 μ g of VR1012 complexed with lipid. By day 15 post tumor cell injection, mice treated with either the 50 or 100 μ g dose of VR1110 complexed with lipid had a significant inhibition of tumor growth ($p=0.002$) compared to the mice treated with the VR1012: lipid (FIG. 18A). A significant increase in survival ($p=0.01$) was also found for the mice treated with either the 50 or 100 μ g dose of VR1110: lipid (FIG. 18B). On day 25, none of the mice treated with the VR1012: lipid survived, while the mice injected with 50 or 100 μ g of VR1110: lipid had 27 and 33% survival, respectively. By day 37, mice treated with 50 or 100 μ g of VR1110: lipid had 20 and 27% survival, respectively. Tumor-bearing mice treated with 25 μ g of VR1110 complexed with lipid were not significantly different from the control mice for either tumor volume or survival.

Cytokine profile of ovarian tumor ascites

Since i.p. injection of IL-2 pDNA: lipid into mice bearing i.p. MOT tumors resulted in high levels of IL-2 expression in the ascites, it was of interest to determine whether the IL-2 therapy initiated a cytokine cascade in

the tumor ascites. C3H/HeN mice were injected i.p. with 10^5 MOT cells. On days 5, 8 and 11 after tumor cell implant, the mice were injected i.p. with either VR1110: lipid or VR1012: lipid (1:1 pDNA:DMRIE mass ratio) or received no treatment after the MOT tumor cell injection. Two days after each injection of pDNA: lipid (days 7, 10 and 13 after tumor cell implant), 5 mice per group were sacrificed and the tumor ascites was collected. The total volume of ascites was determined per mouse. The ascites samples were spun at 14,000 rpm for 2 minutes followed by collection of the supernatants. The ascites supernatants were assayed for the concentration of the cytokines: IL-2, IL-4, IL-6, IL-10, IL-12, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma ($\text{IFN}\gamma$) and tumor necrosis factor alpha ($\text{TNF}\alpha$) using ELISA (R & D Systems, Minneapolis, MN). The concentration of transforming growth factor beta ($\text{TGF}\beta$) in the ascites was assayed using the $\text{TGF}\beta_2$ Emax Immunoassay System (Promega, Madison, WI). The amount of cytokine in the tumor ascites was calculated using the formula: cytokine concentration in pg/ml x ml of total ascites = pg of cytokine/total ascites.

As expected, tumor-bearing mice injected i.p. with VR1110: lipid had a marked increase in IL-2 levels with negligible levels in untreated tumor-bearing mice or mice injected with the VR1012: lipid (FIG. 19A). The levels of $\text{IFN}\gamma$ and GM-CSF were also markedly elevated in the mice treated with VR1110: lipid (FIGs. 19B and 19C). The $\text{IFN}\gamma$ and GM-CSF levels in these mice increased on days 10 and 13 after tumor cell injection but not on the day 7 timepoint suggesting that this could be due to IL-2 secretion. Some non-specific increase in $\text{IFN}\gamma$ was also found in the ascites of tumor-bearing mice after injection of the VR1012: lipid; however, by day 13, the levels of $\text{IFN}\gamma$ in the tumor-bearing mice treated with VR1110: lipid were 6-fold higher than in the mice treated with VR1012: lipid suggesting that expression of IL-2 upregulates $\text{IFN}\gamma$ production.

Levels of IL-6, $\text{TNF}\alpha$ and IL-10 were increased in both the IL-2 pDNA: lipid group as well as the control pDNA: lipid group suggesting that pDNA: lipid complexes may non-specifically stimulate production of these

particular cytokines in the tumor ascites (FIGs. 19D, 19E, and 19F). No differences were found for IL-4, IL-12 or TGF β in the ascites from any of the groups and levels of these cytokines were low (0-300 pg/ml for IL-4 and IL-12 and 0-2000 pg/ml for TGF β , data not shown). For all of the cytokines
5 evaluated, mice treated with control pDNA without lipid, IL-2 pDNA without lipid or with lipid alone had similar cytokine levels as the untreated mice.

Intraperitoneal injection of IFN α pDNA:lipid enhances survival

10 C3H/HeN mice were injected i.p. with 10⁵ MOT cells to establish ovarian i.p. tumors. The mice then received i.p. injections of 100 μ g of VR4111 (mIFN α) or VR1012 (control) complexed with DMR1E:DOPE cationic lipid at a 1:1 DNA:DMR1E mass ratio in a total volume of 1 ml saline. The mice received the i.p. injections of pDNA: lipid on days 5, 8 and
15 11 after tumor cell injection. The mice were weighed 3-6 times per week. Fifteen mice were included in each treatment group.

Mice bearing i.p. MOT tumors and treated with i.p. VR4111: lipid had a significant increase in survival ($p < 0.006$) compared to the mice receiving the control plasmid (FIG. 20B). No significant reduction in tumor volume was
20 found for the VR4111: lipid-treated mice (FIG. 20A).

Example 7

25 ***Selective Transfection of Malignant Cells in Murine Intraperitoneal Melanoma Tumor Model***

The anti-tumor effect of DNA formulations with or without lipids in mouse i.p. melanoma model have been evaluated in the present example.

30

Cell line and tumor model

B16F10 mouse melanoma cells were grown *in vitro* in DMEM and 10% FCS. Two hundred thousand B16F10 mouse melanoma cells were
5 implanted i.p. in C57BL/6 mice in 1-3 ml saline using a 28 G 1/2 needle and without puncturing internal organs.

Preparation of plasmid DNA:lipid complexes

10 The plasmid DNA-cationic lipid formulations were prepared just prior to use. Equal volumes of DNA and DMRIE : DOPE (1:1) were mixed by swirling to achieve a target concentration of 0.5 mg DNA/ml, 100 µg DMRIE/ml and 0.12 mg DOPE/ml as previously described (Parker et al, 1996; Saffran et al, 1998). The other cationic lipids were mixed similarly so that the
15 mass ratio of DNA to cationic lipid was 5:1 and the cationic lipid to DOPE molar ratio was 1:1. The formulated material was then vortexed at high speed for 30 seconds and kept at room temperature until dose administration.

CAT assay

20 Tumor or other tissues were collected, immediately frozen in liquid nitrogen and ground into a powder using a reversible drill as described (Manthorpe, M., Hartikka, J., Vahlsing, H.L. and Sawdey, M. Quantification of plasmid DNA transfection in vivo. In Gene Quantification. F. Ferre, ed.
25 Birkhauser, Boston, Ma., 1998 in press.). The dry frozen powder was thawed and extracted in lysis buffer and high speed supernates assayed for CAT activity using a two-phase partition assay as described (Sankaran, L., Analytical Biochemistry 200: 180-186 (1992)).

Cationic lipids enhances tumor transfection

C57BL/6 mice were injected i.p. with 200,000 B16F10 murine melanoma cells, and seven days later, injected i.p. with CAT pDNA (VR1332):DMRIE/DOPE. Two days later, tumor tissues were collected, extracted, and assayed for CAT activity.

As shown in Table 4, DNA alone transfected tumor, but DMRIE:DOPE increased transfection by 78 fold (from 2,326 to 182,052).

Table 4

pgs CAT per gm of tumor tissue collected (n = 4 to 6 mice as indicated); saline values subtracted			
Mouse #	DNA only	DNA:DMRIE	DMRIE, no DNA
1	0	82,600	0
2	307	137,551	0
3	706	220,600	0
4	791	287,458	0
5	5,892		0
6	6,258		
Average	2,326	182,052	0
Std Error	1,306	52,140	0
Fold higher	1	78	0

Selective transfection of tumor cells

Normal animal. Normal, non-tumor bearing BALB/c mice were injected i.p. with 1mg/2ml VR1332, with or without a cationic lipid, and with or without the neutral lipid, DOPE. Two days later, selected i.p. tissues (liver, lung, kidney, spleen, mesentery) were collected, extracted, and assayed for CAT activity.

As shown in Table 5, normal intraperitoneal organs were not at all transfected or transfected very little with a variety of cationic lipids: There was a low level transfection of mesentery tissues, and a moderate transfection of 1 of 5 spleens (the one transfected spleen may have been punctured by the injection needle).

Table 5

Average pgs CAT per gm of tumor tissue (avg., n = 5 mice)				
Saline background values have been subtracted				
ORGANS	DNA only	+ DMRIE	+ β AE-DMRIE	+ GAP-DLRIE
Liver	0	0	0	0
Lung	0	0	0	0
Kidney	0	0	0	0
Spleen	0	2,563(0)*	0	0
Mesentery	370	1,549	1,866	1,127

ORGANS	+ DOSPA	+ DMRIE, no DOPE	+ β AE-DMRIE, no DOPE
Liver	0	0	0
Lung	0	0	0
Kidney	0	0	0
Spleen	0	0	0
Mesentery	600	1,091	3,604

*one mouse with a CAT value; the rest = 0

Tumor-bearing animal compared to normal animal. C57BL/6 mice were injected i.p. with 200,000 B16F10 murine melanoma cells, and seven days later, injected i.p. with VR1332 with or without cationic lipid/DOPE. Two days later, selected i.p. tissues (liver, lung, kidney, spleen, mesentery) were collected, extracted, and assayed for CAT activity.

As shown in Table 6, tumor tissues were transfected at high levels with pDNA complexed with cationic lipid/DOPE. Normal i.p. organs were not transfected well compared to i.p. tumor tissues.

Table 6

Average pgs CAT per gm of tumor tissue (n = 5 mice); saline values subtracted

ORGANS	DNA only	DNA + DMRIE:DOPE	DNA + DMRIE:DOPE normal mice no tumors
Tumor	0	200,195	n/a
Liver	0	0	0
Lung	0	0	0
Kidney	0	0	0
Spleen	493	0	0
Mesentery	6,795	nd	2,562
Ovary	nd	nd	0

5

Dose Response

C57BL/6 mice were injected i.p. with 200,000 B16F10 murine melanoma cells, and seven days later, injected i.p. with 0.15 or 1.5 mgs of VR1332 with or without cationic lipid/DOPE in 3 ml saline. Two days later, tumor tissues were collected, extracted, and assayed for CAT activity.

10

As shown in Table 7, a higher dose of DNA transfected tumor tissues better than a lower dose. Also, DMRIE transfected better than the two other cationic lipids tested.

Table 7

Average pgs CAT per gm of tumor tissue (n = 5 mice); saline values subtracted

DNA DOSE	DNA only	+ DMRIE	+ GAP-DMRIE	+ PA-DELO
1.5 mgs	16,592	1,270,466	524,006	581,616
0.15 mgs	1,937	131,089	47,866	99,797

5 ***Testing a variety of cationic lipids***

C57BL/6 mice were injected i.p. with 200,000 B16F10 murine melanoma cells, and seven days later, injected i.p. with 1 mg of VR1332 with or without cationic lipid/DOPE in 3 ml saline. Two days later, tumor tissues
10 were collected, extracted, and assayed for CAT activity.

As shown in Table 8, all cationic lipids tested increased transfection level, and DMRIE was one of three preferred cationic lipids.

Table 8

Average pgs CAT per gm of tumor tissue (n = 5 mice);
saline values have been subtracted

CL:DOPE	Run 1	Run 2	Average
None	6,247	not done	6,247
GAP-DDRIE	20,816	7,027	13,922
GMU-DMRIE	9,355	52,888	31,122
HP-DORIE	36,615	26,795	31,705
DOSPA	33,494	57,026	45,260
PA-TELO	63,326	32,681	48,004
GA-LOE-BP	59,729	63,528	61,629
GAP-DMRIE	80,760	63,002	71,881
PA-DELO	73,692	82,265	77,979
GAP-DLRIE	77,553	107,629	92,591
DMRIE	77,128	122,225	99,677
DLRIE	122,999	128,225	125,612
PA-DEMO	155,369	154,884	155,127

In sum, the success of the intra-cavity delivery embodiment of the present invention is also exemplified in the murine melanoma tumor model.

- 5 Following i.p. injection of a polynucleotide, transfection occurs predominantly in tumor tissues, and normal intraperitoneal organs, such as liver, lung, and kidney are poorly transfected, if at all, with the polynucleotide formulation.

- 10 The following examples demonstrate the surprising finding that compositions comprising polypeptide-encoding polynucleotides and certain salts and/or auxiliary agents can enhance subsequent gene expression when administered into murine tissues.

Materials and Methods

The following materials and methods apply generally to all the examples disclosed herein. Specific materials and methods are disclosed in
5 each example, as necessary.

Preparation of the pharmaceutical compositions

All salts used in the following examples are available from Sigma
10 Chemical Corporation (Sigma, St. Louis, MO). Detergents used in the following examples are available from Sigma, Roche Molecular Biochemicals (Indianapolis, IN), BASF (Mount Olive, NJ), and Amresco (Solon, OH). Purified plasmid DNA was ethanol precipitated and resuspended in water. Salt solutions were prepared as 300 mM stock solutions and dilutions were
15 made using sterile USP water (Baxter, Deerfield, IL).

Preparation of plasmid DNAs

Figure 21 depicts the major structural and regulatory elements
20 contained in each plasmid. The gene for *Photinus pyralis* (firefly) luciferase was subcloned from the pSP-LuC vector (available from Promega, Madison, WI) into the VR1012 vector (Manthorpe, M., *et al.*, *Hum. Gene Ther.* 4:419-431 (1993)) to make VR1223 or VR1255 (Hartikka, J., *et al.*, *Hum. Gene Ther.* 7:1205-1217 (1996)). The RSV promoter-regulated VR1418 LacZ
25 vector was made by subcloning the LacZ gene from the VR1412 vector (Doh, S.G., *et al.*, *Gene Ther.* 4:648-663 (1997)) into VR1043, itself derived by replacing the CMV control elements of VR1012 with RSV control elements. The mouse erythropoietin (EPO) was obtained by PCR as described (Tripathy, S.K., *et al.*, *Proc. Natl. Acad. Sci. USA* 93:10876-10880 (1996)) and
30 subcloned into the VR1012 vector to produce VR2901. The secreted form of the human placental alkaline phosphatase (SEAP) gene was subcloned from pSEAP2-Basic (available from Clontech, Palo Alto, CA) into the VR1012

backbone vector to make VR3301. The rat preproinsulin coding sequence was obtained from reverse transcription of rat pancreatic preproinsulin poly(A) mRNA as described (Abai, A.M., *et al.*, *Hum. Gene Ther.* 10:2637-2649 (1999)) and subcloned into the VR1012 backbone vector to produce VR3502.

5 The human IFN- ω coding sequence was obtained by amplifying the coding sequence from human genomic DNA prepared from DNA of fresh human blood. The mouse IFN- α gene was a generous gift from Paula Pitha-Rowe (Johns Hopkins University). The IFN- ω and IFN- α genes were subcloned into the VR1055 vector to produce, respectively VR4151 and VR4111 as described
10 (Horton, H.M., *et al.*, *Proc. Natl. Acad. Sci. USA* 96:1553-1558 (1999)). The luciferase gene in VR1255 was replaced with the influenza A/PR/8/34 nucleoprotein gene as described (Ulmer, J.B., *et al.*, *Ann. N.Y. Acad. Sci.* 772:117-125 (1995)) to yield VR4700.

15 *Plasmid DNA purification*

Plasmid DNA was transformed into *Escherichia coli* DH5 α competent cells and highly purified covalently closed circular plasmid DNA was isolated by a modified lysis procedure (Horn, N.A., *et al.*, *Hum. Gene Ther.* 6:565-573
20 (1995)) followed by standard double CsCl-ethidium bromide gradient ultracentrifugation (Sambrook, J., *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Plainview, New York (1989)). Alternatively, SEAP and preproinsulin encoding plasmid DNAs were purified using Giga columns from Qiagen (Valencia, CA) according to the kit
25 instructions. All plasmid preparations were free of detectable chromosomal DNA, RNA and protein impurities based on gel analysis and the bicinchoninic protein assay (Pierce Chem. Co., Rockford IL). Endotoxin levels were measured using *Limulus* Amebocyte Lysate assay (LAL, Associates of Cape Cod, Falmouth, MA) and were less than 0.6 Endotoxin Units/mg of plasmid
30 DNA. The spectrophotometric A₂₆₀/A₂₈₀ ratios of the DNA solutions were typically above 1.8. Plasmids were ethanol precipitated and resuspended in

water at 4°C until completely dissolved. DNA was stored at -20°C until use. DNA was diluted by mixing it with 300 mM salt solutions and by adding appropriate amount of USP water to obtain 1 mg/ml plasmid DNA in the desired salt at the desired molar concentration.

5

Injections of plasmid DNA

The quadriceps muscles (or tibialis anterior muscles for SEAP plasmids) of restrained awake mice (female 6 - 12 week old BALB/c or Nude,
10 *nu/nu*, from Harlan Sprague Dawley, Indianapolis, IN) were injected with 50 µg of DNA in 50 µl solution using a disposable sterile, plastic insulin syringe and 28G 1/2 needle (Becton-Dickinson, Franklin Lakes, NJ, Cat. No. 329430) fitted with a plastic collar cut from a micropipette tip, all as previously described (Hartikka, J., *et al.*, *Hum. Gene Ther.* 7:1205-1217 (1996)). The
15 tissues were extracted and assayed as described in Manthorpe, M., *et al.*, "Quantification of plasmid DNA expression *in vivo*," in *Gene Quantification*, Ferre, F., ed., F. Birkhäuser, Boston, MA (1998), pp. 343-368. Briefly, tissues were rapidly collected and frozen, they were combined with and frozen into a lysis buffer, the tissue was then pulverized with a drill bit run in reverse
20 direction, the resulting powder was thawed, and was extracted two times with extraction buffer. Nude mice were injected with VR3301 DNA bilaterally on three consecutive days for a total of 300 µg DNA per mouse. Lungs were instilled with 132 µg plasmid DNA complexed with GAP-DLRIE/DOPE in solution and extracted all as described (Wheeler, C.J., *et al.*, *Proc Natl Acad Sci USA* 93:11454-11459 (1996)).
25

Animal care throughout the study was in compliance with the "Guide for the Use and Care of Laboratory Animals", Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press, Washington, D.C., 1996 as well as with Vical's
30 Institutional Animal Care and Use Committee.

Enzyme assays

Luciferase activity was assayed using a Dynatech model ML2250 microplate luminometer (Chantilly, VA) as previously described (Hartikka, J.,
5 *et al.*, *Hum. Gene Ther.* 7:1205-1217 (1996)). The luciferase content of the samples was calculated from Relative Light Units using a standard curve of purified firefly luciferase (Analytical Luminescence Laboratory, Sparks, MD, Cat. No. 2400), which was diluted in pooled extract from uninjected muscles to control for quenching. Luciferase values were expressed as ng luciferase
10 per muscle. The level of β -galactosidase expression in muscle extracts was quantified using a chemiluminescent assay according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN, Cat. No. 1758241). A standard curve, prepared in pooled extract from uninjected muscles, was included on each plate using the β -galactosidase enzyme
15 standard included in the kit.

Sera from nude mice injected with VR3301 were collected at various times post-injection. Five μ l of serum was mixed with 15 μ l distilled H₂O and placed into individual wells of a 96 well plate (e.g., Costar EIA/RIA A/2 #3690, available from Corning, Inc). A serial dilution of EIA grade calf
20 intestinal alkaline phosphatase (CIP, available from Roche Molecular Biochemicals, cat. # 567 744) in PBS containing 0.05% Bovine Serum Albumin (BSA) was used to produce a standard curve. Samples were assayed in duplicate. Plates were sealed, incubated at 65°C for 30 min and spun for 5 min at 4000 rpm at room temperature. Each well received 100 μ l substrate
25 solution containing 1 mg/ml para-nitrophenyl phosphate (PNPP, available from Roche Molecular Biochemicals, cat. # 107 905) and 1mM MgCl₂ in 1M diethanolamine, pH 9.8. The samples were analyzed using a Molecular Devices Opti Max plate reader (Sunnyvale, CA). The plate reader was pre-warmed to 37°C and a standard kinetic program was used to assay the samples
30 at a wavelength of 405 nm for 30 min.

NP, proinsulin, and β -galactosidase ELISA

Sera were collected at different times before and after plasmid DNA injections. Ninety-six well plates (available from Corning Incorporated, Acton, MA, Cat. No. 3690) were coated with 36 ng/50 μ l of Borate Buffered Saline (BBS) /well of NP purified from recombinant baculoviral extracts (available from Imgenix Corporation), commercial rat proinsulin (available from Crystal Chem, Chicago, IL) or 5 μ l / 50 μ l of β -galactosidase protein (available from Sigma). The plates were stored overnight at +4°C and the wells washed twice with Borate Buffered Saline Tween (BBST) (89 mM Boric Acid + 90 mM NaCl, pH 8.3 + 234 mM NaOH supplemented with 0.05 % Tween 20® (v/v)). The wells were then incubated 90 minutes with BB (BBST in which the Tween was replaced with 5% non-fat milk in 1X BBS) and washed twice with BBST again. Two-fold serial dilutions of mouse serum in BB starting at 1:20 were made in successive wells and the solutions were incubated for 2 hours at room temperature. Wells were then rinsed four times with BBST. Sera from mice hyperimmunized with VR4700 NP, VR3502, or VR1412 plasmid DNA were used as a positive control and pre-immune sera were used as negative controls.

To detect specific antibodies, alkaline phosphatase conjugated goat anti-mouse IgG-Fc (*e.g.*, from Jackson Immunoresearch Laboratories, Inc. West Grove, PA, Cat. No. 115-055-008) diluted 1: 5000 in BBS were added at 100 μ g/well and the plates were incubated at room temperature for 2 hours. After 4 washings in BBST, the substrate (1 mg/ml p-nitrophenyl phosphate, *e.g.*, from Calbiochem-Novabiochem Corp., San Diego, CA, Cat. No. 4876 in 50 mM sodium bicarbonate buffer, pH 9.8 and 1 mM MgCl₂) was incubated for 90 min at room temperature and absorbance readings were performed at 405 nm. The titer of the sera was determined by using the reciprocal of the last dilution still giving a signal two times above background. Background was established using pre-immune serum diluted 1:20. Serum concentrations

of human IFN- ω were measured using a commercially available kit with a detection limit of 2 pg/ml (available from Alexis Corp., San Diego, CA).

Hematocrit measurements

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Hematocrits were measured by centrifugation of blood obtained from the retro-orbital cavity of mice. Blood samples were collected in 75 μ l heparinized capillary tubes and analyzed using HemaSTAT II microhematocrit centrifuge (Separation Technology, Inc., Altamone Springs, FL).

10

Histology

For whole muscle staining, quadriceps were fixed for 3 hours at room temperature in 2% paraformaldehyde in PBS, washed 3 times for 20 min each in PBS and incubated for 18 hours at 37 °C in a solution containing 2 mM MgCl₂, 5 mM potassium ferricferrocyanide and 1 mg/ml 5-bromo-4-chloro-3-indoyl- β -D-galactosidase (available from Life Technologies, Inc. (LTI), Gaithersburg, MD) in PBS. After incubation, the muscles were washed 3 times for 10 min each in 3% dimethyl sulfoxide in PBS and stored in PBS at 4°C until analysis. To prepare stained tissue cross sections, quadriceps were snap-frozen in liquid nitrogen-cooled isopentane, cut in half, embedded in OCT medium (available from VWR, McGraw Park, IL) and 10 μ m sections were cut using a Jung Frigocut Model 2800E cryostat (Leica, Foster City, CA). Sections were collected on 1% gelatin coated glass slides, brought to room temperature and stained for 2 hours at 37°C in the same reagent as for whole mounts above except that the beta-galactosidase reagent concentration was 200 μ g/ml. The sections were then counterstained with Harris hematoxylin in acetic acid, rinsed in tap water, dehydrated and mounted in Permout (Fisher, Fair Lawn, New Jersey). The number of β -galactosidase positive cells per muscle was determined by light microscopy in muscle cross-sections as described (Doh, S.G., *et al.*, *Gene Ther.* 4:648-663 (1997)).

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Splenocyte CTL stimulation cultures

To generate CTL effector cells from plasmid DNA immunized
5 BALB/c mice, splenocytes were stimulated in culture for 5 days with NP₁₄₇₋₁₅₅
peptide (TYQRTRALV) pulsed, irradiated splenocytes from naïve BALB/c
mice. For the stimulation cultures, splenocytes from naïve mice were γ -
irradiated with 3200 Rads and pulsed with 10 μ M of the H-2K^d restricted
NP₁₄₇₋₁₅₅ peptide for 40 min at 37°C. Then, 2.5×10^7 test splenocytes from
10 DNA immunized mice were incubated at 37°C in 5.5% CO₂ with an equal
number of irradiated, pulsed splenocytes from naïve mice in 25 cm² flasks
containing 25 ml RPMI 1640 media with L-glutamine and 25 mM HEPES
supplemented with 10% fetal bovine serum, penicillin (100 U/ml),
streptomycin (100 μ g/ml) and 5.5×10^{-5} M β -mercaptoethanol). Tissue culture
15 reagents are all available from LTI. After 24 hours of culture, recombinant
murine IL-2 was added to a final concentration of 1 U/ml.

⁵¹Cr release assay

20 To measure specific lysis of NP₁₄₇₋₁₅₅ peptide pulsed target cells by
CTL effector cells, P815 cells (available from the American Type Culture
Collection, Manassas, VA) were labeled with Na₂⁵¹CrO₄ (NEN Life Scientific
Products, Inc., Boston, MA). Aliquots of ⁵¹Cr labeled cells were either pulsed
with 10 μ M NP₁₄₇₋₁₅₅ or were used unpulsed. For the CTL assay, stimulated
25 splenocytes were serially diluted in 96 well round bottom microtiter plates
(available from ICN Biomedicals, Inc., Aurora, OH). Target cells were added
in a final volume of 100 μ l/well. After incubation for 5 hours at 37°C in 5.5%
CO₂, 100 μ l of RPMI 1640 complete media was added to each well, the plates
were centrifuged and 100 μ l/well was removed for analysis in a Cobra II
30 gamma counter (Packard Instruments Co., Downers Grove, IL). The
percentage of specific lysis was calculated as % specific lysis = $(a-b/c-b)100$

where a is the average cpm released in the presence of effectors, b is the average spontaneous cpm released from target cells incubated in media only and c is the maximum cpm released from target cells in the presence of 1% Triton X-100™.

5

DNase inhibition assay

Quadriceps muscles were harvested from naïve mice as previously described (Hartikka, J., *et al.*, *Hum. Gene Ther.* 7:1205-1217 (1996)). Ten
10 muscles were pooled and ground at 4°C without adding buffer using a Duall tissue grinder with ground glass pestle (Kontes Glass Co., Vineland, NJ), centrifuged at 13,000 x g for 20 min, and the supernatant was collected and stored at -20°C. Serum was collected from 10 naïve mice by orbital sinus
15 puncture, pooled and stored at 4°C. For the assay, VR1255 DNA was diluted to 0.25 µg/ml in the respective solution, mixed with 0.1 volume of muscle extract or serum, and incubated at 37°C for 2 hours. The samples were neutralized by adding an equal volume of 2% sodium dodecyl sulfate + 50 mM EDTA, and 4 µl of each sample was analyzed by electrophoresis on 0.8% agarose Tris Acetate EDTA gels with ethidium bromide staining.

20

Statistical evaluations

All statistical comparisons from tissue expression data were performed using the non-parametric Mann-Whitney rank sum test (SigmaStat version
25 2.03, Jandel Scientific Software, San Rafael, CA) and where indicated by the standard Student T-Test. Differences by all statistical methods were considered statistically significant when the p value was less than 0.05.

Example 8

Effect of Various Solutions Containing Sodium Chloride and Sodium Phosphate on Luciferase Plasmid DNA Expression in Murine Muscles

5 The purpose of the present example is to demonstrate the ability of certain salt solutions to increase the levels of plasmid DNA expression when injected into muscle compared with plasmid DNAs formulated in normal saline.

10 Mouse quadriceps muscles were injected with 50 μ g of plasmid VR1223, encoding luciferase, dissolved in 50 μ l of either water, saline, PBS, saline plus 100 mM sodium phosphate, 100 mM NaCl, or 100 mM NaCl plus 50 mM sodium phosphate. The muscles were extracted and assayed for luciferase activity 7 days later. The results are shown in Table 9. When the plasmid was dissolved in distilled water, luciferase expression was 25-times
15 lower than when the plasmid was dissolved in saline (4 vs. 119 ng lux/muscle). Injection of the plasmid dissolved in PBS (*i.e.*, saline plus 10 mM sodium phosphate) elicited a marginal, but statistically higher 1.6-fold expression level than saline (186 vs. 119 ng luciferase per muscle, $p = 0.02$). Delivery of the plasmid in a hypertonic solution containing saline plus 100
20 mM sodium phosphate reduced expression to the level obtained using saline alone (117 vs. 119 ng lux/muscle). A hypoosmotic 100 mM NaCl solution yielded the same expression as isoosmotic saline (112 vs 119 ng lux/muscle), but restoration of osmolarity by the addition of 50 mM sodium phosphate to the 100 mM NaCl increased expression by 1.8 fold (112 vs 203 ng lux/muscle,
25 $p = 0.03$). Thus, sodium phosphate increased luciferase expression.

Example 9

Effect of the Molar Concentration of Sodium Phosphate and pH on Luciferase Plasmid DNA Expression in Murine Muscles

5 The effect of sodium phosphate concentration and pH on the level of
luciferase expression from injected plasmid DNA in murine muscles was
tested as follows. Plasmid VR1223 DNA was dissolved in solutions
containing different concentrations of sodium phosphate in the *absence* of
NaCl, and these were tested for day 7 luciferase expression in quadriceps
10 muscle as described in Example 1 above. The molar concentrations of sodium
phosphate tested ranged from 2.5 mM to 300 mM. The averaged data from 5
separate experiments are shown in Figure 22A. Peak expression occurred
when the plasmid DNA was dissolved in 150 mM sodium phosphate, which
yielded 386 ng luciferase per muscle which is 4.3-fold higher than the average
15 expression level observed when the DNA is dissolved in saline (indicated by
the dashed line at 89 ng luciferase per muscle, $p < 0.001$). The expression
levels observed when the DNA was dissolved in 80 mM, 100 mM, 150 mM,
and 200 mM sodium phosphate solutions were significantly higher than saline
by Mann-Whitney rank sum test ($p < 0.05$). Injection of plasmid DNA
20 dissolved in solutions having sodium phosphate concentrations below 40 mM
(in the absence of added chloride ion) or above 300 mM resulted in luciferase
expression levels equal to or lower than those seen with saline.

To examine the effect of pH, plasmid VR1223 was dissolved in 150
mM sodium phosphate or potassium phosphate at pHs of 6.5, 7.5 or 8.0 and
25 was tested for day 7 expression in quadriceps muscle as described in Example
1 above. The results indicated that an optimal pH of about 6.5 to 7.5, with pH
8.0 being suboptimal (Figure 22B).

Example 10

Effect of Alternate Salt Solutions on Luciferase Plasmid DNA Expression in Murine Muscles

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In this example, injection of plasmid DNA encoding luciferase dissolved in 150 mM solutions of various salts which vary either the cation or the anion of normal saline were compared with saline for their ability to stimulate luciferase expression in murine muscle. The results are shown in Table 10.

Table 10A shows the effect on luciferase expression when the plasmid DNA is dissolved in a solution of a salt where the sodium cation in saline is replaced with other cations. Plasmid VR1223 dissolved in the various solutions was tested for day 7 expression in quadriceps muscle as described in Example 1 above. Two salts, ZnCl_2 and FeCl_2 , were only tested in 2 or 3 mice since these salts appeared to cause pain. Solutions containing divalent cations, e.g., magnesium (Mg^{2+}), calcium (Ca^{2+}), zinc (Zn^{2+}) and ferrous iron (Fe^{2+}), greatly decreased expression while the solution containing the monovalent cation potassium (K^+) elicited the same expression as the monovalent sodium cation (Na^+).

Table 10B shows the effect on luciferase expression when the plasmid DNA is dissolved in a solution of a salt where the sodium cation in sodium phosphate was replaced with various other cations. Plasmid VR1223 dissolved in the various solutions was tested for day 7 expression in quadriceps muscle as described in Example 1 above. Just as replacing the sodium cation in saline with potassium cation did not affect luciferase expression, replacing the sodium cation in sodium phosphate with potassium cation also had no effect. Thus, a solution of 150 mM potassium phosphate stimulated expression just as well as solution of 150 mM sodium phosphate when both were compared with saline. When plasmid DNA was dissolved in 150 mM solutions of dibasic or monobasic sodium phosphate (not adjusted for pH), luciferase expression was

only slightly stimulated over saline. The best stimulation of expression occurred when the plasmid DNA was dissolved in a 150 mM solution of sodium or potassium phosphates which is a mixture of the dibasic and monobasic forms balanced to achieve the desired pH (in this case, pH 7.0).

- 5 Other phosphate salts tested, *i.e.*, when the cation was Mg^{3+} , Ca^{3+} , Aluminum (Al^{3+}) or ferric iron (Fe^{3+}) resulted in inhibited expression relative to saline.

Table 10C shows the effect on luciferase expression when the plasmid DNA is dissolved in a solution of a salt where the phosphate anion in 150 mM sodium phosphate was replaced with various other anions. Plasmid VR1223 dissolved in the various solutions was tested for day 7 expression in quadriceps muscle as described in Example 1 above. Injection of the plasmid DNA in solutions of the sodium salts of acetate, pyruvate, bicarbonate and sulfate all increased luciferase expression compared with saline. Sodium citrate yielded the same luciferase expression as saline but sodium oxalate inhibited luciferase expression. Thus, according to Table 10C, stimulatory effects of various 150 mM salt solutions can be ranked in order of their relative enhancement of luciferase expression as follows: sodium phosphate = potassium phosphate = sodium acetate > sodium pyruvate = sodium bicarbonate = sodium sulfate > saline = potassium chloride = sodium citrate.

20 The rest of the solutions tested inhibited expression compared with saline.

The effects of osmolarity and pH on the ability of certain salt solutions to enhance luciferase expression in murine muscle was tested as follows. The osmolarity and pH of each salt solution (150 mM concentration unless otherwise indicated) shown in Tables 11-A and 11-B were measured and plotted vs. the 7- day luciferase expression level obtained with that solution (Figures 22C and 2D). The pH values and osmolarities of the various salt solutions, as well as the relative 7-day luciferase expression observed when plasmid VR1223 was dissolved in each solution and injected into murine muscle, are shown in Table 11-A and 11-B. The osmolarity graph (Figure 22D) revealed that solutions with osmolarities between 271 and 349 mmol/kg generally yielded the highest expression levels but exceptions included 50 & 100 mM sodium phosphate at 83 and 270 mmol/kg (respective expression

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levels 2.1 and 2.3 fold those of saline at 310 mmol/kg) and sodium citrate at 394 mmol/kg (expression level was the same as with saline). The 150 mM sodium phosphate solution yielded an expression level that was 4-fold higher than that of saline, yet both solutions had the same osmolarity (310 vs. 308 mmol/kg, respectively). The pH graph (Figure 22C) revealed that the highest expression levels were generally obtained with solutions at pH 6.0 to 7.5. However, some exceptions were sodium sulfate, pH = 5.5, and sodium bicarbonate, pH 9.0, which yielded expression levels that were 2.6 and 2.8-fold, respectively, over saline at pH 5.5. Furthermore, the magnesium phosphate solution had a pH = 7.0 but yielded an expression level lower than saline.

The reproducibility of enhanced luciferase DNA expression when the plasmid DNA is dissolved in a solution of 150 mM sodium phosphate was tested as follows. Nine different experiments were carried out to test luciferase expression levels when plasmid VR1223 was dissolved in 150 mM sodium phosphate for injection into mouse skeletal muscle. In each of the experiments, 10 quadriceps muscles were injected with 50 µg of plasmid VR1223 in 50 µl of either saline or 150 mM sodium phosphate. Muscles were collected and assayed for luciferase expression at 7 days. The averaged results for each experiment are shown in Figure 23. Compared with saline, sodium phosphate enhanced luciferase expression in all 9 experiments, with the enhancement ranging from 2.5-fold (156 vs. 384 ng lux/muscle for Exp. # 9) to 7.3-fold (49 vs. 362 ng lux/muscle in Exp. # 1). In these replicate experiments, the average enhancement by sodium phosphate for all 9 experiments was 4.1-fold (120 vs. 490 ng luciferase/muscle).

Example 11

Expression of β -galactosidase and Human Interferon-omega Following Intramuscular Injection of Plasmid DNA is Enhanced When the Plasmid is Injected in a Sodium Phosphate Solution

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The effect of a sodium phosphate solution on the expression of polypeptides other than luciferase following intramuscular injection of plasmid DNAs encoding the polypeptides was examined as follows. Plasmids encoding non-secreted β -galactosidase (VR1418) and secreted human
10 interferon-omega (IFN- ω ; VR4151) in saline or 150 mM sodium phosphate were injected into mouse quadriceps as described in the materials and methods and Example 1. Muscle extracts were assayed for β -galactosidase and the serum was assayed for circulating levels of IFN- ω . The 7 day post-DNA injection protein expression data, including luciferase plasmid DNA run in
15 parallel for comparison, are shown in Figure 24. Injection of the plasmid DNAs dissolved in 150 mM sodium phosphate enhanced expression of all three proteins over saline. Compared with saline, injection of plasmid DNA in 150 mM sodium phosphate enhanced expression of luciferase in muscle by 4.8-fold (769 vs. 159 ng/muscle; $p < 0.001$), β -galactosidase in muscle by 3.3-
20 fold (9.8 vs. 3.0 ng/muscle; $p = 0.001$) and serum IFN- ω levels by 2.5-fold (0.35 vs. 0.14 ng ml serum; $p = 0.020$).

Plasmid DNA dissolved in PBS, run in parallel, elicited statistically equivalent expression as saline for the plasmids encoding β -galactosidase and IFN plasmids (data not shown).

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Plasmid DNA VR1418 encoding β -galactosidase was dissolved in sodium phosphate solutions at the various molar concentrations tested in Example 2. Quadriceps muscles were injected with 50 μ l of each solution containing 10 μ g of plasmid VR1418, and the muscles were tested for 7-day expression levels. As with luciferase expression, as shown in Example 2, 150
30 mM sodium phosphate was the optimal molar concentration for β -galactosidase expression (data not shown). This similar effect occurred

despite the fact that the β -galactosidase gene on VR1418 is driven by an RSV promoter, rather than the CMV promoter which drives expression of luciferase on plasmid VR1223 and IFN- ω on plasmid VR4151, and despite the fact that 10 μ g of plasmid DNA was injected per muscle rather than 50 μ g.

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Example 12

Expression of Human Placental Alkaline Phosphatase, Rat Proinsulin and Mouse Erythropoietin Following Intramuscular Injection of Plasmid DNA is Enhanced When the Plasmid is Injected in a Sodium Phosphate Solution

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Three different plasmid DNAs encoding a secreted form of human placental alkaline phosphatase (SEAP; VR3301), rat preproinsulin (VR3502) or mouse erythropoietin (EPO; VR2901) were injected into mouse skeletal muscle in saline or 150 mM sodium phosphate solutions as described in the Materials and Methods and in Example 1. The mice were monitored over time for blood levels of SEAP or proinsulin or for hematocrits. The results are shown in Figure 25. In the case of SEAP, plasmid DNA was injected into the tibialis anterior muscles, and nude mice were used to prevent an immune response to the foreign transgene product. The kinetics of blood SEAP levels from mice injected with DNA dissolved in both saline and 150 mM sodium phosphate were similar. SEAP protein expression rose to a peak level at 7 days, then declined to 40-45% of the maximum expression level where it remained for two months. The Sodium phosphate solution significantly enhanced SEAP expression by 1.4 to 1.8-fold compared with saline (n = 15; p 0.002 to 0.037) over the time course. A parallel experiment using PBS showed expression to be statistically equivalent to that obtained with saline (data not shown). Thus, injection of plasmid DNA dissolved in a 150 mM sodium phosphate solution resulted in a higher level of sustained expression than when dissolved in saline.

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Plasmid DNA encoding rat preproinsulin, dissolved in either 150 mM sodium phosphate or saline, was injected into the muscles of immunocompetent mice. Injection of the plasmid DNA in the sodium phosphate solution enhanced expression over a 2 week period by 1.9 to 3.8-
5 fold compared with saline ($n = 10$; $p < 0.01$). Proinsulin expression eventually declined to very low levels in both groups, possibly due to the generation of measurable anti-proinsulin antibodies (data not shown).

Plasmid DNA encoding mouse EPO, dissolved in either 150 mM sodium phosphate or saline, was injected into the muscles of
10 immunocompetent mice. Hematocrit levels, which correlate with the expression of erythropoietin, rose steadily over 4 weeks. Control mice injected with 20 μ g of DNA encoding canine Factor IX exhibited a constant hematocrit averaging 48. Injection of plasmid DNA dissolved in 150 mM sodium phosphate solution resulted in higher hematocrit levels than the injection of
15 plasmid DNA dissolved in saline at all the time points tested. This enhancement ranged from 1.4 to 2.1-fold ($n = 10$; $p = 0.02$ - 0.001).

Example 13

Histological Analysis of Muscle Tissues Injected with Plasmid DNA 20 Encoding β -galactosidase Dissolved in Either Sodium Phosphate or Saline

Individual muscle cells were examined for β -galactosidase expression as follows. Twenty-six BALB/c quadriceps muscles each were injected with 50 μ g of plasmid VR1412 DNA (expressing β -galactosidase) in 50 μ l of
25 either saline or 150 mM sodium phosphate, according to the methods disclosed in the Materials and Methods and in Example 1. The muscles were collected 7 days later and stained for β -galactosidase. A quantitative analysis of β -galactosidase-stained fibers using previously detailed methods (Doh, S.G., *et al.*, *Gene Ther.* 4:648-663 (1997)) revealed a significantly greater
30 number of β -galactosidase-stained myofiber cells in the sodium phosphate group than in the saline group. Cell counts of sections taken from the midline of 20 muscles (10 muscles for each group) revealed that the sodium phosphate

group contained more β -galactosidase-positive myofiber cells than did the saline group (average of 108 +/- 21 vs. 186 +/- 43 myofiber cells/muscle section; $p = 0.02$; $n=10$). Thus, plasmid DNA dissolved in 150 mM sodium phosphate apparently has an enhanced ability to transduce muscle cells relative to plasmid DNA dissolved in saline.

Example 14

Sodium phosphate inhibits muscle DNase activity

While not being bound by theory, the inventors believe that one mechanism by which sodium phosphate could enhance plasmid DNA expression in muscle is by preventing or inhibiting DNA degradation. To assess this possibility, aliquots of plasmid DNA in various aqueous solutions were spiked with mouse muscle extract or serum as described in the materials and methods section, above. The reactions were incubated for 2 hours at 37°C in the presence of water, saline or 150 mM sodium phosphate. After incubation, DNA degradation was analyzed by agarose gel electrophoresis. The results are shown in Figure 26. Incubation of plasmid DNA in water with 10% v/v muscle extracts or serum resulted in the complete degradation of the DNA. Compare lanes 1 vs. 2 in Figure 26. A significant amount of the DNA was protected in the presence of both saline and sodium phosphate. However, the sodium phosphate solution protected the DNA considerably better than did saline. Compare lanes 3 and 4 in Figure 26; note the presence of remaining closed circular form in the muscle extract and the remaining nicked form in the serum for the DNA in the sodium phosphate solution. Similar results were obtained in replicate experiments. This inhibition of DNA degradation correlates with the increase in gene expression observed for DNA injected into muscle (compare ng luciferase expression values taken from Tables I and II and shown at the bottom of each lane in Figure 26 with the presence of nicked and closed circular DNA).

Example 15

Plasmid DNA Immunization Utilizing a Sodium Phosphate Solution

The effect of sodium phosphate on the elicitation of an immune response upon injection of plasmid DNA encoding an immunogen was examined as follows. Mice were vaccinated intramuscularly with plasmid VR4700, encoding the influenza nucleoprotein. The DNA was dissolved in either saline or 150 mM sodium phosphate. The mice were monitored for the presence of circulating anti-NP antibodies and for an NP-specific cytotoxic T lymphocyte response (CTL). The antibody data at 6 weeks and CTL data at 9 weeks post-vaccination are shown in Figure 27. Figure 27A shows that in three replicate experiments (labeled 1-3, n = 10 mice per experiment), the sodium phosphate solution enhanced serum anti-NP antibody titers compared with saline solution. The enhancement by sodium phosphate was significant in all 3 experiments ($p < 0.04$) as well as in the average of all three experiments ($p < 0.001$). Plasmid VR VR4700, injected in PBS, was run in parallel and the antibody titers were not statistically different from the plasmid injected in saline (data not shown). Figure 27B shows that anti-NP specific CTL activity was statistically similar ($p > 0.05$) in the saline and 150 mM sodium phosphate group. A repeat set of CTL experiments showed the same result (data not shown).

Example 16

Polypeptide Expression in a Non-Muscle Tissue is Enhanced by Use of a Sodium phosphate Solution and a Cationic Lipid for Delivery of Plasmid DNA

The effect of a sodium phosphate solution on the enhancement of polypeptide expression from plasmid DNA delivered to a non-muscle tissue using a cationic lipid was evaluated as follows. Mouse lungs were instilled with plasmid VR1223 encoding luciferase combined with the cationic lipid GAP-

DLRIE and co-lipid DOPE in water or selected concentrations of sodium phosphate as described in the Materials and Methods above. The use of DNA lipid complexes in water and collection at day 3 were previously found to yield peak lung transfection (Wheeler, C.J., *et al.*, *Proc Natl Acad Sci USA* 93:11454-11459 (1996); Sawa, T., *et al.*, *Hum. Gene Ther* 7:933-941 (1996)).

5 The results are shown in Figure 8. The level of luciferase expression in lung when the plasmid/lipid mixture was delivered to the lung in water (0.94 ng lux/lung) is comparable with published reports (Wheeler, C.J., *et al.*, *Proc Natl Acad Sci USA* 93:11454-11459 (1996)) and is considerably below the

10 level of expression obtained in muscle tissue using the same vectors without lipid. Unlike with muscle, when the plasmid/lipid mixture was delivered to the lung in a 150 mM sodium phosphate solution, luciferase expression was inhibited compared with water (0.22 vs. 0.94 ng lux/lung; $p < 0.001$).

15 However, when the plasmid/lipid mixture was delivered to the lung in a 2.5 mM sodium phosphate solution, luciferase expression was enhanced by 5.5-fold compared with water (5.2 vs. 0.94 ng lux/lung; $p < 0.001$). Intermediate to these values, when the plasmid/lipid mixture was delivered to the lung in a 10 mM sodium phosphate solution, luciferase expression was enhanced compared with water, but not as much as with 2.5 mM sodium phosphate. When the

20 plasmid/lipid mixture was delivered to the lung in saline, luciferase expression was inhibited compared with water (0.14 ng lux/lung; data not shown). Thus, sodium phosphate enhances luciferase expression upon delivery of a plasmid DNA/lipid mixture in lung, but does so at a much lower sodium phosphate molar concentration than is effective in muscle.

Table 9
Effects of Selected Sodium Chloride Vehicles on Luciferase Plasmid DNA
expression in Muscle

ng Lux per Muscle	± Std. Error	n	Fold Saline	Salt Solution
4	1	30	0.03	Double-distilled water
119	6	413	1.0	150 mM Sodium Chloride (saline)
186	11	357	1.6	150 mM Sodium Chloride (saline) + 10 mM Sodium Phosphate (PBS)
117	64	20	1.0	150 mM Sodium Chloride (saline) + 100 mM Sodium Phosphate
112	38	20	0.9	100 mM Sodium Chloride
203	42	40	1.7	100 mM Sodium Chloride + 50 mM Sodium Phosphate

Table 10
Effects of Selected Vehicles on Luciferase Plasmid DNA expression
in Muscle

ng Lux / Muscle	± Std. Error	n	Fold Saline	Salt Solution	Formula
A. Chloride Salts at 150 mM					
119	6	413	1.0	Sodium Chloride	NaCl
124	32	10	1.0	Potassium Chloride	KCl
1	0.4	10	<0.1	Magnesium Chloride	MgCl ₂ · 6H ₂ O
0.3	0.2	10	<0.1	Calcium Chloride	CaCl ₂ · 2H ₂ O
0.1	0.1	6	<0.1	Zinc Chloride	ZnCl ₂
0.0	0.0	4	<0.1	Ferrous Chloride	FeCl ₂ · 4H ₂ O
B. Phosphate salts at 150 mM					
481	36	120	4.0	Sodium Phosphate	NaH ₂ PO ₄ / K ₂ HPO ₄
282	56	20	2.4	" -- dibasic	Na ₂ HPO ₄
198	44	20	1.7	" --monobasic	Na ₂ HPO ₄
449	40	20	3.8	Potassium Phosphate	KH ₂ PO ₄ / K ₂ HPO ₄
22	6	8	0.2	Magnesium Phosphate	MgHPO ₄ · 3H ₂ O
12	2	8	0.1	Calcium Phosphate	CaHPO ₄
4	1	8	<0.1	Aluminum Phosphate	AlPO ₄
0.4	0	10	<0.1	Ferric Phosphate	FePO ₄
C. Sodium salts at 150 mM					
119	6	413	1.0	Sodium Chloride	NaCl
481	36	120	4.0	Sodium Phosphate	NaH ₂ PO ₄ / Na ₂ HPO ₄
498	119	10	4.1	Sodium Acetate	C ₂ H ₃ O ₂ Na · 3H ₂ O
364	64	19	3.1	Sodium Pyruvate	C ₃ H ₃ O ₃ Na
330	47	20	2.8	Sodium Bicarbonate	NaHCO ₂
312	83	10	2.6	Sodium Sulfate	NaSO ₄
90	25	14	< 0.1	Sodium Citrate	C ₆ H ₅ Na ₃ O · 2H ₂ O
15	2	8	< 0.1	Sodium Oxalate	C ₂ O ₄ Na ₂

Table 11-A

Salt	PH	lux
Zinc Chloride	5.0	0.1
Ferrous Chloride	5.0	0.0
Aluminum Phosphate	5.0	4
150 mM NaCl (Saline)	5.5	119
100 mM NaCl	5.5	112
Potassium Chloride	5.5	124
Magnesium Chloride	5.5	1
Calcium Chloride	5.5	0.3
Sodium Chloride	5.5	119
NaP – monobasic	5.5	198
Sodium Sulfate	5.5	312
Calcium Phosphate	6.0	12
Sodium Pyruvate	6.0	364
Ferric Phosphate	6.5	0.4
Sodium Acetate	6.5	498
Sodium Oxalate	6.5	15
Magnesium Phosphate	7.0	22
Saline + 10 mM Na-P (PBS)	7.2	186
75 mM NaCl + 75 mM Na-P	7.2	177
50 mM Sodium Phosphate	7.2	254
100 mM Sodium Phosphate	7.2	270
150 mM Sodium Phosphate	7.2	481
Sodium Phosphate	7.2	481
Potassium Phosphate	7.5	449
Sodium Citrate	7.5	108
50 mM Sodium Citrate	7.5	83
NaP – dibasic	8.0	282
Sodium Pyrophosphate	9.0	3
Sodium Bicarbonate	9.0	330

Table 11-B

Salt	Osm	lux
Calcium Phosphate	36	12
Aluminum Phosphate	37	4
Magnesium Phosphate	39	22
50 mM Sodium Phosphate	83	254
50 mM Sodium Citrate	164	83
Ferric Phosphate	165	0.4
100 mM NaCl	215	112
100 mM Sodium Phosphate	232	270
Sodium Acetate	271	498
Sodium Pyruvate	271	364
Sodium Bicarbonate	727	330
NaP – monobasic	277	198
Potassium Chloride	280	124
150 mM NaCl + 10 mM Na-P (PBS)	292	186
75 mM NaCl + 75 mM Na-P	308	177
150 mM Sodium Phosphate	308	481
150 mM NaCl (Saline)	310	119
Potassium Phosphate	323	449
Sodium Oxalate	346	15
Sodium Sulfate	349	312
NaP – dibasic	357	282
Zinc Chloride	358	0.1
Magnesium Chloride	360	1
Calcium Chloride	362	0.3
Ferrous Chloride	362	0.0
Sodium Pyrophosphate	363	3
Sodium Citrate	394	108

It clear that the invention may be practiced otherwise than as particular described in the foregoing description and examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of
5 the appended claims.

The entire disclosure of all publications (including patents, patent application, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.